DNA Damage Response and Replication Stress in Mouse Embryonic Stem Cells

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ZUSAMMENFASSUNG

Die Replikation des Erbinformationsträgers DNA ist ein zentraler zellulärer Prozess, der sicherstellt, dass das gesamte genetische Material dupliziert wird, bevor die Information auf die beiden Tochterzellen aufgeteilt wird. Die zelluläre Antwort auf DNA Schäden („DNA Damage Response DDR“) schützt die Zellen vor schädlichen Mutationen während der Replikation und stellt die genetische Stabilität der DNA sicher, wenn die Zelle genotoxischen Substanzen ausgesetzt ist. In Stammzellen muss diese zelluläre Antwort auf DNA Schäden besonders robust sein, da diese sich fortwährend teilen und in verschiedene Zelltypen oder Gewebetypen differenzieren können.

Die wichtigste Funktion von embryonalen Stammzellen ist einerseits, Tochterzellen mit Stammzellcharakter zu generieren und andererseits sich in jegliches Gewebe auszudifferenzieren. Der schnelle zeitliche Ablauf des Zellzyklus kommt aufgrund eines schnellen G1-S Überganges und einer hohen Konzentrationen von CDKs und anderen Zellzyklus Regulatoren zustande. In unserer Arbeit konnten wir aufzeigen, dass embryonale Stammzellen von Mäusen erstaunlicherweise endogenem Replikations-Stress ausgesetzt sind. Dieser Replikations-Stress konnte charakterisiert werden durch ein hohes Grundniveau des ATR-abhängigen DDR Marker γH2AX, durch die Rekrutierung der Einzelstrang-bindenden Proteine RPA und Rad51, die Akkumulierung von ssDNA Lücken, durch eine erhöhte Häufigkeit des Phänomens "Umkehrung der Replikations-Gabel" und eine stark reduzierte Einbaurate von Nukleotiden. All diese Replikations-Stress Phänomene gehen durch die Einleitung der Differenzierung verloren, noch bevor die Zellen aufhören, sich zu teilen. Zudem konnten wir zeigen, dass PARP1, ein Faktor beteiligt an der Replikation von beschädigter DNA in somatischen Zellen, in ESCs benötigt wird, um die Integrität des Replikations-Komplexes sicherzustellen. Unsere Hypothese, welche in den nächsten Wochen getestet wird, ist, dass Replikations-Initiationsfaktoren („origin firing factors“) in ESCs geschwindigkeitslimitierend sind, was zur Vererbung von nur teilweise verdoppelter DNA bei schnellem zeitlichen Ablaufes des Zellzyklus führt. Eine Überexprimierung des “Firing Factors” Cdc45 und/oder eine Veränderung der Länge des Zellzyklus durch Inhibierung von CDKs reduziert die DNA-Reparatur-Antwort in ESCs.

Hämatopoetische Stammzellen HSCs oder auch Blutstammzellen sind Ausganspunkt für die gesamte Zellneubildung des Blutes und des Abwehrsystems. HSCs sind meistens ruhende Zellen und werden durch Verletzungen oder Entzündungen aktiviert, um Gewebe

Homöostase zu erreichen. Ruhende HSCs können in Mäusen durch Stimulation von Interferon alpha (IFN-a) aktiviert werden. Vorversuche dieser Studie weisen darauf hin, dass die meisten aktivierten HSCs erhöhte γH2AX Levels aufweisen. Diese Resultate geben zu verstehen, dass HSCs, welche aus dem Ruhezustand austreten, Replikations-Stress Symptome aufweisen, ähnlich wie sich aktiv teilende ESCs.

Die Haupt-Erkenntnisse dieser Arbeit legen nahe, dass DDR in sich aktiv teilenden Stammzellen eine nicht-vollständige Replikation signalisiert.

SUMMARY

DNA replication is a central cellular process that allows duplication of the genetic material before its proper segregation during cell division. The DNA damage response (DDR) protects cells from deleterious mutations during replication and helps maintain genome stability in face of exogenous genotoxic stress. Such pathways must be particularly robust in stem cells, since they are constantly self-renewing and capable of differentiating into all other specialized cells.

The main function of embryonic stem cells (ESCs) is to proliferate and differentiate into multiple cell types spatiotemporally, without compromising on their self-renewal capacity. The high proliferative capacity of ESCs is often coupled to rapid G1-S transition and elevated levels of CDKs and other cell cycle regulators. In this study, we show that mouse ESCs surprisingly experience endogenous DNA replication stress (RS), which is characterized by high basal levels of the ATR-dependent DDR marker γH2AX, chromatin recruitment of the single stranded DNA (ssDNA) binding proteins RPA and Rad51, accumulation of ssDNA gaps/nicks, increased replication fork reversal and slow fork progression. Strikingly, all these hallmarks of RS are quickly lost upon induction of differentiation, before cells stop proliferating. Furthermore, PARP1 activity - previously shown to be involved in replication of damaged DNA in somatic cells - is required to protect replication fork integrity in unperturbed ESCs. Our working hypothesis, which will be directly addressed in the next weeks, is that origin firing factors are rate limiting in ESCs, leading to inheritance of partially replicated DNA during fast cell cycle progression. Indeed, overexpression of the firing factor Cdc45 and/or altering the cell cycle length by inhibiting CDK activity, reduces DDR signalling in ESCs.

Haematopoietic stem cells (HSCs) possess the ability to give rise to all the cells of the haematopoietic system. HSCs are mainly quiescent and are activated upon injury or inflammation to bring about tissue homeostasis. Stimulation of mice with interferon alpha (IFN-α) specifically activates dormant HSCs. Preliminary observations in this study suggest that most "activated" HSCs exhibit elevated γH2AX staining. These results suggest that HSCs that exit from dormancy may experience RS, similarly to actively proliferating ESCs.

Collectively, the main findings in this study suggest that the active DDR in proliferating stem cells signals incomplete replication inherited during fast cell cycle progression.

1. INTRODUCTION

1.1 Eukaryotic DNA replication

Cellular proliferation is an essential process during the development and maintenance of an organism. It is tightly regulated and is controlled at various points during the lifetime of an individual. As cells divide, so does their genome. The precise duplication of DNA and its segregation into daughter cells is of prime importance. The basic machinery that is employed during semi-conservative DNA replication is conserved from prokaryotes to eukaryotes. To ensure fidelity during DNA replication, the co-ordinated action of various factors is paramount. This process is organized into three distinct phases: initiation, elongation and termination.

1.1.1 Initiation of DNA replication

Replication initiates at specific sites located on the genome called origins. In lower eukaryotes, origin sites have been efficiently mapped for some species, e.g. budding yeast (Wyrick et al., 2001) owing to clear consensus sequences. This, however, is more challenging in higher eukaryotes. Although origin sequences are not clearly defined in mammals, they have been associated with certain features, which help predict potential replication initiation sites. For instance, AT rich sequences (Paixao et al., 2004, Altman and Fanning, 2004, Wang et al., 2004a) and matrix attachment regions (Schaarschmidt et al., 2003, Debatisse et al., 2004) have been consistently linked with initiation. Several reports also demonstrate topology of DNA to play in important role in origin selection (Remus et al., 2004, Houchens et al., 2008, Abdurashidova et al., 2007). Other parameters such as distal elements (Aladjem et al., 1995, Hayashida et al., 2006) and chromatin structure (Burke et al., 2001, Prioleau et al., 2003, Besnard et al., 2012) have also been shown to govern origin choice.

The first step in initiation is binding of the origin recognition complex (ORC) to a DNA sequence (Shackleton et al., 1992). In mammals, ORC does not have any apparent sequence specificity for the region of DNA it binds (Vashee et al., 2003, Schaarschmidt et al., 2003). After ORC binding, Cdc6, Cdt1 and the hexameric MCM2-7 complex are sequentially loaded onto chromatin (Fig 1) between late mitosis and early G1 to generate the pre-replicative complex (preRC) (Masai et al., 2010). It has been shown that preRC assembly is a

prerequisite but is not sufficient to determine replication initiation. In other words, preRCs mark potential origins but only a subset are licensed for use during each round of replication (Edwards et al., 2002, Hyrien et al., 2003). The phosphorylation of Mcm2 by Cdc7 is necessary for licensing of replication origins at least in the G0 to S transition context (Chuang et al., 2009). The kinase activity of Cdc7 is also essential later during replication (see 1.1.2) (Donaldson et al., 1998, Bousset and Diffley, 1998). Although licensing may appear to be stochastic, reports have clearly demonstrated that origin choice is reproducible at least after addition of hydroxyurea (HU) in yeast (Feng et al., 2006, Hayashi et al., 2007). Cdc6 and Cdt1 have been shown to play a role in replication licensing and ensure that each origin is used only once per cell cycle (Rowles et al., 1999, Maiorano et al., 2000). Both Cdc6 and Cdt1 are regulated in a cell cycle dependant manner to prevent re-replication (see 1.2.2.3). Cdc6 destruction during S phase occurs via Cdk2 phosphorylation (Duursma and Agami, 2005) whereas Cdt1 is inactivated via ubiquitin-dependant proteolytic degradation or by a specific inhibitor of Cdt1, Geminin (Nishitani et al., 2006, Xouri et al., 2007). Geminin itself is then degraded in mitosis and is absent in the following G1 phase to allow for origin licensing (McGarry and Kirschner, 1998). However, origin licensing is differentially regulated in embryonic stem cells (ESCs), where Geminin escapes degradation during the G1 phase and is required for maintenance of pluripotency (Yang et al., 2011a). In contrast, origins are licensed more frequently in ESCs and origin distribution is re-organized only upon differentiation (Hiratani et al., 2008). Further, licensing factors are much more abundant in ESCs and their levels drop during differentiation (Fujii-Yamamoto et al., 2005). These aspects of DNA replication in ESCs distinguishes them from other somatic cells and have been further elaborated in chapter 1.3.1.3.

1.1.2 Origin firing and chain elongation

The conversion of a preRC complex into initiation complex (IC) is a critical step required for replication. This step is executed upon phosphorylation by two kinases- cyclin dependant kinase (CDK) and Dbf4 dependant Cdc7 kinase (Masai and Arai, 2002, Sclafani, 2000). CDK and Cdc7 mediated activation of preRC allows for loading RPA, MCM10 and Cdc45 onto chromatin to bring about origin firing (Zhu et al., 2007). Further, the budding yeast Sld2 and Sld3 in conjunction with Dpb11 interact with Cdc45 and are also required for origin firing (Tanaka et al., 2007). Indeed, the respective orthologues of yeast Sld2 and Sld3 in higher

eukaryotes, namely RecQL4 and Treslin, have been shown to be essential for origin firing (Gaggioli et al., 2014, Kumagai et al., 2010). Hence, origin firing is regulated by co-ordination between various factors. Not all origins that are licensed are eventually fired. Studies have shown that origin firing (and not licensing) is rate limiting in eukaryotes (Wu and Nurse, 2009, Patel et al., 2008, Yoshida *et al., in press*). Further, firing is differentially regulated during early embryogenesis (Collart et al., 2013). This is further discussed in chapter 1.2.2.3.

A novel factor that assists in chain elongation, GINS, is necessary for the stable association of Cdc45 with the MCM complex in S phase (Gambus et al., 2006). Ctf4 and MCM10 coordinate with GINS and DNA polymerase alpha (pol α) to promote fork progression (Stillman, 2008). The helicase activity of the MCM complex is required to unwind the DNA duplex and the ssDNA generated is stabilized by RPA, following which pol α synthesises a 30 nucleotide long RNA/DNA primer. Subsequently, the replication fork complex (RFC) binds to the primer and loads PCNA which assists in switching pol α for more processive DNA polymerases, pol δ and/or pol ε. Thereafter, replication elongation takes place on the leading strand via RFC, PCNA and pol ε and on the lagging strand where the Okazaki fragments are extended by RFC, PCNA and pol δ (Hübscher et al., 2002, Garg and Burgers, 2005, Stillman, 2008).

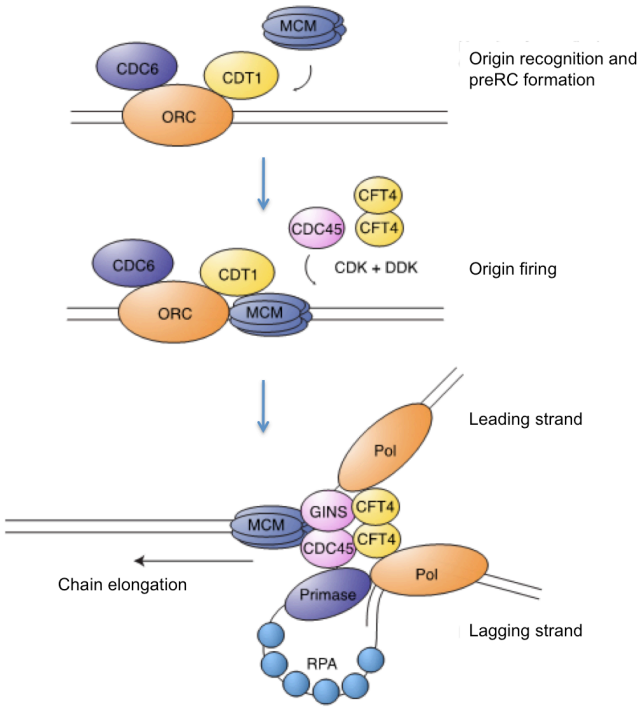


Figure 1: Replication initiation, origin firing and chain elongation. Illustration representing different steps in eukaryotic DNA replication. Origin recognition and formation of the preRC complex comprises binding of ORC to DNA and subsequent recruitment of the Mcm2-7 helicase complex by Cdc6 and Cdt1. Firing of origins is brought about by the loading of Cdc45 and other firing factors and requires CDK and DDK activity and (see text). Subsequently, ORC, Cdc6 and Cdt1 dissociate from DNA and replication chain elongation occurs by the co-ordinated action of polymerases and other components of the replication complex. Modified from (Sørensen et al., 2011).

1.1.3 Replication termination

Very little is known about termination of replication in eukaryotes. Most of our knowledge comes from studies on plasmid replication in *Xenopus* egg extracts or yeast chromosomal replication. Since multiple origins are fired on each chromosome, termination occurs when forks converge between two origins or upon encountering telomeric sequences at chromosomal ends (Santamaria et al., 2000). Certain site-specific replication barriers can also act as replication terminators. Replication barriers were first discovered in the *S. cerevisiae* rDNA (Linskens and Huberman, 1988, Brewer and Fangman, 1988, Brewer et al., 1992), namely RFB1 and RFB2. Fob1, the factor required for replication termination in yeast, has been shown to interact with RFB1 and RFB2 *in vitro* (Mohanty and Bastia, 2004). Fob1 acts to prevent collision between the replication and transcription machineries (see 1.2.2.5) (Takeuchi et al., 2003). In mammals, the transcription factor TTF1 acts as a fork barrier at rDNA (Little et al., 1993). Ku70/Ku 86 have also been reported to possess replication termination activity (Wallisch et al., 2002). It has been proposed in this study that Ku70/Ku86, in collaboration with TTF1, bind to the Sal box 2 and stabilize secondary structures that are formed either by unwinding of the DNA by the helicase or due to replication by the polymerase to facilitate termination. Further, certain termination regions (TERs) have been identified in eukaryotes where replication forks are known to pause. The DNA helicase Rrm3 helps in fork progression through these regions whereas the DNA topoisomerase Top2 brings about fork fusion thereby preventing genomic instability at TERs (Fachinetti et al., 2010).

1.2 DNA replication stress

The replication machinery is quite robust and can tolerate most impediments that might arise during the course of the process. However, deregulation of internal cellular processes or external sources that may interfere with replication can cause cells to experience replication stress (RS) (see 1.2.2). As discussed in the previous section, every cell fires only a subset of origins during replication, suggesting that surplus origins may be redundant. However, in the face of RS leading to fork stalling, dormant origins are fired to allow replication to be completed (Ibarra et al., 2008). Of course, there are pathways that help cells deal with RS

(1.2.1). But when such pathways malfunction, this leads to replication catastrophe (Chanoux et al., 2009). Hence, for DNA replication to proceed uninterrupted and in an accurate manner, several factors need to be controlled.

1.2.1 Sensing RS and responding to it

So far, RS has not been sufficiently characterized and its definition is still evolving; this is mainly due to the absence of unambiguous markers that can be safely used to detect this phenomenon. A defining feature of RS is slowing or stalling of replication forks and/or DNA synthesis (Zeman and Cimprich, 2014). RS may be largely uncoupled from DNA double strand break (DSB) formation as has been shown at least in three different scenarios that lead to DNA damage if RS is not dealt with (Chaudhuri et al., 2012). However, many sources of RS (see 1.2.2) can cause formation of single stranded (ss) DNA stretches that result from unwinding of parental DNA even after the polymerase has stalled (Pacek and Walter, 2004). These regions of ssDNA in the vicinity of a stalled replication fork are coated by replication protein-A (RPA), which signals the RS response pathway (Byun et al., 2005). The central kinase that senses RS, ataxia telangiectasia mutated and Rad3 related (ATR), is then activated and thereby allows the cell to complete replication in the face of stress (Zou and Elledge, 2003, MacDougall et al., 2007, Edward and David, 2011). It must be noted that not all sources of RS lead to long ssDNA formation. Along the same line, RS is not necessarily associated with ATR dependant phosphorylation of RPA and CHK1. A few forks may be stalled and elicit a response locally, while a global response is not necessary (Koundrioukoff et al., 2013).

The ATR pathway acts to stabilize forks in response to RS. When the source of RS is removed, stalled forks can be restarted (Petermann and Helleday, 2010). In cases where the source cannot be removed, i.e. when the DNA lesion is unrepaired, there are other pathways in place that help fork restart. There are two possible ways to tackle such a problem- either by firing dormant origins (Woodward et al., 2006, Ge et al., 2007, McIntosh and Blow, 2012) or by re-priming the replication machinery downstream of the lesion (Lopes et al., 2006, Elvers et al., 2011). Both outcomes prevent prolonged fork stalling and allow replication to continue (Fig 2). In the latter scenario, the restart of replication leaves behind a ssDNA gap that is filled by the DNA damage tolerance (DDT) pathway. The DDT pathway employs specialized polymerases or makes use of the sister chromatid to allow for tolerating or bypassing the

lesion (Mailand et al., 2013). In spite of several checks to promote unhindered replication, stalled forks may collapse when they cannot be restarted. This happens especially when the source of RS persists or when factors involved in fork protection are absent (Lopes et al., 2001, Tercero and Diffley, 2001, Cobb et al., 2003), and the problem is exacerbated when ATR itself is lost (Chanoux et al., 2009). As a gross consequence there are many diseases that are associated with prolonged replication stress, the most common being cancer (Negrini et al., 2010).

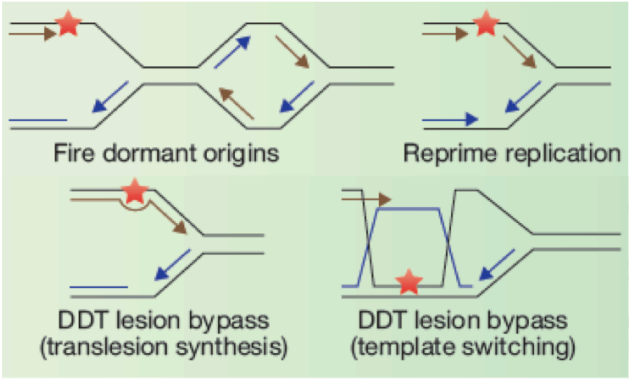


Figure 2: Mechanisms of fork restart. Illustration of key intermediates in replication fork restart. Forks that stall at DNA lesions (indicated by the red star) can restart replication either by firing dormant origins or by repriming DNA synthesis. DNA lesions can also be bypassed via the DNA damage tolerance (DDT) pathways. Modified from (Zeman and Cimprich, 2014)

1.2.1.1 Factors involved in fork protection

What is now becoming increasingly clear is that proteins that were known only to be involved in the homologous recombination (HR) repair pathway (see 1.3.4) also serve to protect stalled forks from collapsing. Rad51 has been shown to prevent accumulation of ssDNA at and behind the replication fork, independent from its role in HR (Hashimoto et al., 2010). The tumour suppressor gene BRCA2 assists in Rad51 loading during HR (Esashi et al., 2007), but prevents stalled forks from being degraded irrespective of its HR function (Schlacher et al., 2011). In addition, core Fanconi anaemia (FA) proteins have been consistently linked with DNA inter strand cross-link repair but reports in the past decade have shown that the FA network is also activated upon RS due to depletion of nucleotide pools (see 1.2.2.4) where

physical lesions do not occur (Howlett et al., 2005, Naim and Rosselli, 2009). Indeed, BRCA2 has been shown to functionally interact with the FA gene FANCD2 (Hussain et al., 2004, Wang et al., 2004b). This interplay between FA and HR players has recently been demonstrated to be essential for fork stabilization and once again underscores the importance of Rad51 filament formation in preventing fork degradation, uncoupling it from its activity in HR (Schlacher et al., 2012). Further, the resolution of stalled forks via the FA/BRCA pathway requires γH2AX, and FANCD2-H2AX interaction has been suggested to pave the road for lesion bypass via the DDT pathway (Lyakhovich and Surralles, 2007).

1.2.1.2 γH2AX as a marker of RS

The histone variant H2AX has a conserved phosphatidylinositol 3-OH-kinase (PI3K) related kinase motif and is a known target of ataxia telangiectasia mutated (ATM), ATR and DNA dependant protein kinase catalytic subunit (DNA-PKcs) kinases. Its phosphorylated form (serine 139) is known as γH2AX. Traditionally, γH2AX was described in the context of DNA DSBs, where ATM is the major kinase that phosphorylates it (Rogakou et al., 1998). Upon irradiation, γH2AX is formed within minutes, rapidly spreads along the chromatin, and acts a docking platform for other DSB repair proteins (Rogakou et al., 1999, Paull et al., 2000). In addition, γH2AX is also observed during apoptosis when DNA fragmentation is initiated (Rogakou et al., 2000). Interestingly, a landmark study demonstrated that H2AX phosphorylation is independent of ATM in the context of RS induced by HU or UV. Both insults do not generate DSBs and, in such a setting, ATR was identified as the sole kinase responsible for γH2AX (Ward and Chen, 2001). Therefore, although γH2AX alone cannot be used as a marker for RS, when combined with other approaches such as RPA staining and DNA fiber assay (see methods) it can be used as readout for RS (Edward and David, 2011, Bianco et al., 2012, Maréchal and Zou, 2013).

1.2.2 Known sources of RS

There are several sources of RS (Fig 3) out of which those that are relevant for the scope of this thesis will be discussed.

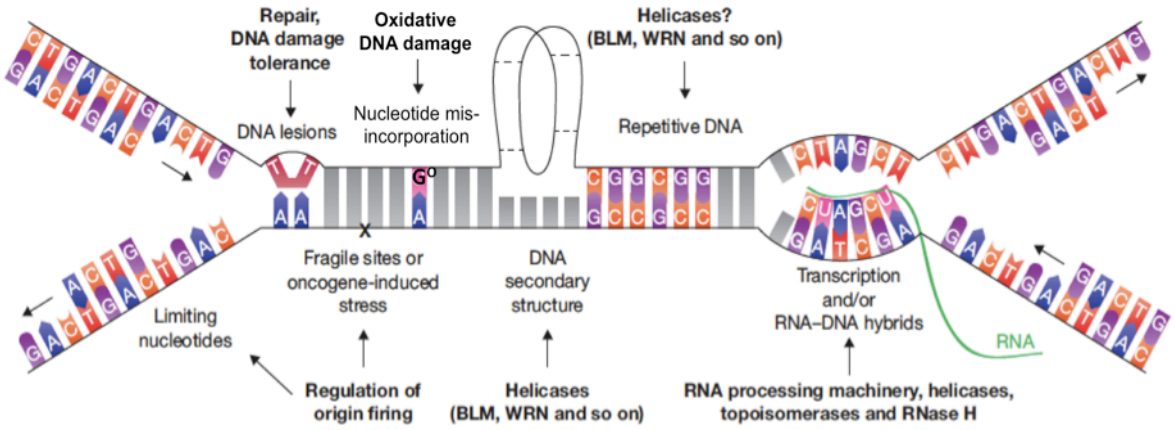


Figure 3: Sources of replication stress. A number of different sources can cause replication fork slowdown or stalling such as regulation of origin firing, DNA lesions, oxidative DNA damage, interference between transcription and replication etc. Most of these are discussed in the text below. Modified from (Zeman and Cimprich, 2014)

1.2.2.1 Chemotherapeutics

One of the hallmarks of cancer is its high proliferative capacity (Hanahan and Weinberg, 2000). The topological stress that is built during replication and transcription in both normal and cancer cells is removed by an enzyme called topoisomerase 1 (Top1) (Koster et al., 2010). This aspect of replication is exploited in the treatment of cancer - i.e. Top1 inhibitors are employed in clinics to slow down disease progression (Pommier, 2006). Camptothecin (CPT) and its derivates belong to the family of Top1 inhibitors and are among the best studied drugs used in chemotherapy (Hsiang et al., 1989). Recent evidence from our lab shows that sub-lethal doses of CPT (Chaudhuri et al., 2012) and other chemotherapeutics (Zellweger et al., manuscript in preparation) can induce RS in cancer cells. Under these conditions, fork slowdown and reversal (see 1.2.3.1) can largely be uncoupled from chromosomal breaks and only when high micromolar doses are used does the RS results in DNA DSBs (Chaudhuri et al., 2012).

1.2.2.2 Oncogene activation

Many oncogenes have been reported to cause RS when activated (Mailand et al., 2000, Bartkova et al., 2005). In early stages of tumorigenesis, RS is known to activate the DNA damage response (DDR) (Bartek et al., 2007). DDR activation in precancerous lesions induces senescence, which prevents malignant transformation (Bartkova et al., 2006, Di Micco et al., 2006). In addition, oncogene activation, either directly or indirectly, causes deregulation of origin licensing and impairs replication fork progression: this can result from depletion of the nucleotide pool (Bester et al., 2011), topological stress (Bermejo et al., 2012), or from collision between replication and transcription (Jones et al., 2013). The slowing down or remodeling of the replication fork are detected quite early during overexpression of at least two oncogenes- Cyclin E and Cdc25. Importantly, at these time points the oncogene overexpression itself does not elicit a full DDR and only when RS persists due to prolonged overexpression of the oncogenes, DSB formation is observed (Neelsen et al., 2013a).

1.2.2.3 Deregulated/altered origin activity

Licensing of replication origins is a tightly regulated process and its deregulation can have dire consequences. Oncogene overexpression (see above) is often associated with supernumerary origin licensing (Hook et al., 2007). Which event precedes the other is often difficult to determine since excess origin firing can be both a cause and consequence of tumour development (Blow and Gillespie, 2008). RS due to over licensing has also been described in other settings besides cancer onset (Beck et al., 2012). On the flipside, insufficient licensing of origins can also lead to RS due to inability of the fork to travel through long distances, especially in difficult to replicate regions (Letessier et al., 2011).

Origins must fire only once per cell cycle. However, when either of the licensing factors Cdc6 or Cdt1 are overexpressed, cells undergo re-replication- i.e., the same origin is fired twice during a single S phase (Vaziri et al., 2003). This is also recapitulated when a negative regulator of origin licensing, Emi1, is downregulated. Once again, RS is detected quite early when Emi1 is depleted and only when re-replicating forks bump into the ssDNA gaps left behind from the first round of deregulated replication do chromosomal breaks occur (Neelsen et al., 2013b).

The individual components of replication initiation can also be rate limiting, especially in a system where replication is paramount- for e.g., during early embryogenesis in *Xenopus laevis*, overexpressing Cut5, Treslin, Recq4, Drf2 causes extra cell divisions at the mid-blastula transition (Collart et al., 2013). In mouse ESCs, either depleting or inhibiting Cdc7 activity, which is required for preRC activation and origin firing, leads to apoptosis (Kim et al., 2002). Cdc45, the factor required for origin firing, has been shown to be rate limiting for replication origin usage. It is present at a much lower level in comparison to the preRC components in mammalian cells. Indeed, excessive Cdc45 activates otherwise dormant origins (Wong et al., 2011) and concomitantly, Cdc45 overexpression leads to replication stress via increased origin usage and subsequently causes DNA damage (Srinivasan et al., 2013). The histone deacetylases Sir2 and Rpd3 serve as replication initiation factors at ribosomal DNA in budding yeast. Deletion of Sir2 and Rpd3 leads to global replication initiation defects, which are completely rescued by overexpressing factors required for replication origin firing in yeast namely Sld3, Sld7 and Cdc45 (Yoshida *et al., in press*). Put together, there is increasing evidence pointing towards replication firing factors being limiting during replication. On the other hand, knocking down the Mcm2-7 complex, which is part of preRC, does not seem to have an effect on fork progression- suggesting that very low amounts of the helicase is sufficient and therefore does not limit origin licensing. Only in the face of replication stress, where dormant origins are fired, does Mcm knockdown have deleterious effects (Ibarra et al., 2008).

1.2.2.4 Nucleotide deficiency

Nucleotides are the building blocks for DNA synthesis and their titration is one of the key aspects during replication. However, nucleotides often become the limiting factor and induce RS especially during early stages of tumorigenesis or due to deregulated origin activity- both of which may be interdependent (see above). The deficit in nucleotide levels is a consequence of hyper origin activation, since the available pool is quickly used up. Addition of hydroxyurea (HU), which inhibits ribonucleotide reductase, also leads to nucleotide exhaustion (Young and Hodas, 1964). When local nucleotide pools are depleted, as in the case of oncogene activation, the genome is under threat and it has been shown that exogenous addition of nucleotides rescues replication fork progression and genome stability (Bester et

al., 2011). Whether supplying nucleotides exogenously can help relieve RS in general is currently a matter of debate.

1.2.2.5 Collision between transcription and replication

In eukaryotes, replication and transcription occur simultaneously on the same DNA molecule. Therefore, interference between the two machineries is inevitable. Indeed, collision between replication and transcription resulting from topological hindrance or R-loop formation is a known source of RS (Bermejo et al., 2012, Aguilera and García-Muse, 2012). In mammalian cells, replication pausing or stalling has been reported to occur preferably in transcribed regions in Top1 depleted cells (Tuduri et al., 2009). Overexpression of Cyclin E is also associated with increased interference between replication and transcription, which is followed by chromosomal breakage and eventually leads to genome instability (Jones et al., 2013). It has also been reported that certain regions of the genome known as common fragile sites (CFS) are sensitive to replication stress. CFS are prone to breakage when replication is slowed down at these loci, as in the case of long genes spanning several kilobases, and is one of the causative factors of malignant transformation (Helmrich et al., 2011).

1.2.2.6 Oxidative DNA damage and hypoxia

Oxidation of DNA bases is a common phenomenon and can occur spontaneously either due to the abundant free radicals present in the cell or as a by-product of various biochemical processes. The most frequent base damage is caused by hydroxyl radicals, which possess the highest reactivity amongst the reactive oxygen species (ROS) (Cooke et al., 2003). Guanine is commonly oxidized to 8-Oxo-G by ROS and as a result, adenine is misincorporated opposite it instead of cytosine (Michaels et al., 1992). If unrepaired, these mispaired bases are often the cause of mutational load in cells (Wang et al., 1998) and are a known cause of RS and tumorigenesis (Jackson et al., 1998). Indeed, MSH2 and MSH6, components of the mismatch repair (MMR) pathway help in removal of the mispaired bases to order to ensure genome stability (Ni et al., 1999).

Hypoxia is defined as inadequate supply of oxygen and is a known cause of tumorigenesis (Rankin and Giaccia, 2008). During angiogenesis, severe hypoxia can cause cessation of DNA synthesis and hence replication fork stalling. This activates the ATR pathway and is characterized by γH2AX foci formation, which helps recruit repair factors and protects endothelial cells from DNA damage (Economopoulou et al., 2009).

1.2.3 Hallmarks of RS at the DNA level

A combination of common features underlies RS, regardless of its source. Some of these have already been described (ssDNA gaps, γH2AX foci formation, replication fork slowdown, RPA recruitment etc.). However, there is a peculiar hallmark of RS at the DNA level - replication fork reversal - that needs to be further elaborated.

1.2.3.1 Fork reversal

Accumulation of ssDNA gaps and reversed forks have been consistently observed while characterizing RS from at least three diverse sources discussed above, namely chemotherapeutics, oncogene activation and deregulated origin activity (Chaudhuri et al., 2012, Neelsen et al., 2013a, Neelsen et al., 2013b). Although there might be interplay between the two events, it is not entirely clear if one event precedes the other or how these structures are exactly formed. Replication fork reversal can be defined as the conversion of a typical replication fork (three-way junction) into a four-way junction by coordinated annealing of the two newly synthesized strands and re-annealing of the parental strands, to form a fourth "regressed" arm at the fork elongation point (Neelsen and Lopes, *in press*). In the aforementioned studies, fork reversal precedes DSB formation. It is thought that eukaryotes might have evolved this mechanism to allow for replication to be rescued in face of stress. When a problem is encountered by an ongoing fork, remodelling the stalled fork avoids running into the lesion thereby preventing chromosomal breakage. At least in the case of Top1 inhibition, fork reversal is dependant on the enzyme poly(ADP) ribose polymerase 1 or PARP1 (see 1.5.3) without which forks are prone to form DNA DSBs (Chaudhuri et al., 2012). The factors that may be directly responsible for fork reversal include the fork protection factors Rad51, BRCA2, FANCD2 (Schlacher et al., 2012) or the annealing

helicases SMARCAL1 and ZRANB3 (Bétous et al., 2012, Weston et al., 2012) and are currently under investigation in the Lopes lab.

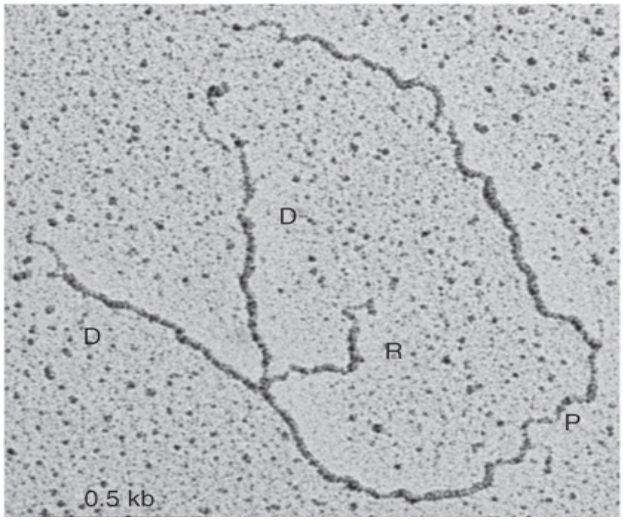


Figure 4: Reversed replication fork visualized by electron microscopy. Fork reversal occurs via formation of a shorter regressed arm, which can be distinguished from parental DNA and newly replicated daughter DNA strands. D = daughter strand, P = parental strand, R = regressed arm. Modified from (Berti et al., 2013).

1.3 Stem cells

The population of cells with the ability of giving rise to other specialized cells while maintaining their self-renewal capacity are called stem cells. Stem cells can be divided into two broad classes- embryonic stem cells (ESCs) and adult stem cells (ASCs). ESCs are pluripotent, i.e., they can give rise to the three germ layers ectoderm, endoderm and mesoderm - almost all the cells in the body. ASCs are largely multipotent, i.e., they can generate only a specific subset of related cell types. For instance, haematopoietic stem cells (HSCs) possess the ability to differentiate into all other blood cells. An important difference between ESCs and most ASCs lies in their proliferative capacity - ESCs divide rapidly, whereas ASCs are usually quiescent and proliferate upon specific stimuli. Thus, even though both populations of stem cells can give rise to differentiated cells, they are very different in terms of their behaviour.

1.3.1 Embryonic stem cells

1.3.1.1 Early embryonic development

Post fertilization, the zygote undergoes a series of mitotic divisions. While dividing, the zygote travels to the uterus and the journey takes three to four days in mice and five to seven days in humans (Fig 5). The first cleavage gives rise to the two-cell stage and further asynchronous divisions produce 4, 8, 16 cells and so on. At the 8-cell stage, the embryo compacts where all the cells are tightly interconnected by gap junctions. This is when the anterior-posterior patterning of the embryo is established and by the 16 cell stage, known as the *morula*, the *trophectoderm* is formed by the outer layer of cells whereas the inner layer forms the *inner cell mass*. What follows then is physical separation of the trophectoderm from the inner cell mass, which is when the *morula* becomes a *blastocyst*. A structure known as *zona pellucida* protects the blastocyst and is only removed upon its implantation into the uterus. Once the pre-implantation embryo hatches, physiological changes occur and various metabolic pathways are activated which allow the development of the embryo into an entire organism. The cells that occupy the inner cell mass of the blastocyst give rise to the three germ layers, whereas the trophectoderm gives rise to the extra-embryonic tissues that form the placenta (source: http://stemcells.nih.gov/).

1.3.1.2 ESC isolation and cultivation

Given the size and accessibility of mammalian embryos, studying cellular aspects of embryogenesis can be quite challenging. Cultivated ESCs are capable of differentiating into all cell lineages and when transplanted into the blastocyst, they participate in normal embryonic development. ESCs can also be genetically modified to study fundamental molecular processes. Hence, the use of ESCs has revolutionized studies in the field of developmental biology.

ESCs are derived from the inner cell mass of the blastocyst (Fig 5). Once isolated, there are several ways of cultivating ESCs *ex vivo*. The traditional way has been to culture ESCs on a 'feeder' layer. The feeder cells generally used are inactivated mouse embryonic fibroblasts (MEFs), which continue to produce leukaemia inhibitory factor (LIF). LIF is a pre-requisite for mouse ESC cultivation and an additional amount is exogenously added to sustain ESC proliferation and prevent their spontaneous differentiation. This method of cultivation makes

use of standard fetal calf serum (for full details, see materials and methods). Serum contains both pro and anti 'stemness' factors, i.e., factors that signal to maintain stem cell properties and those that induce their differentiation. Hence, another way to culture ESCs is using serum replacement medium in which, as the name suggests, media contains sera factors that stimulate stemness and lacks those factors that specifically promote differentiation.

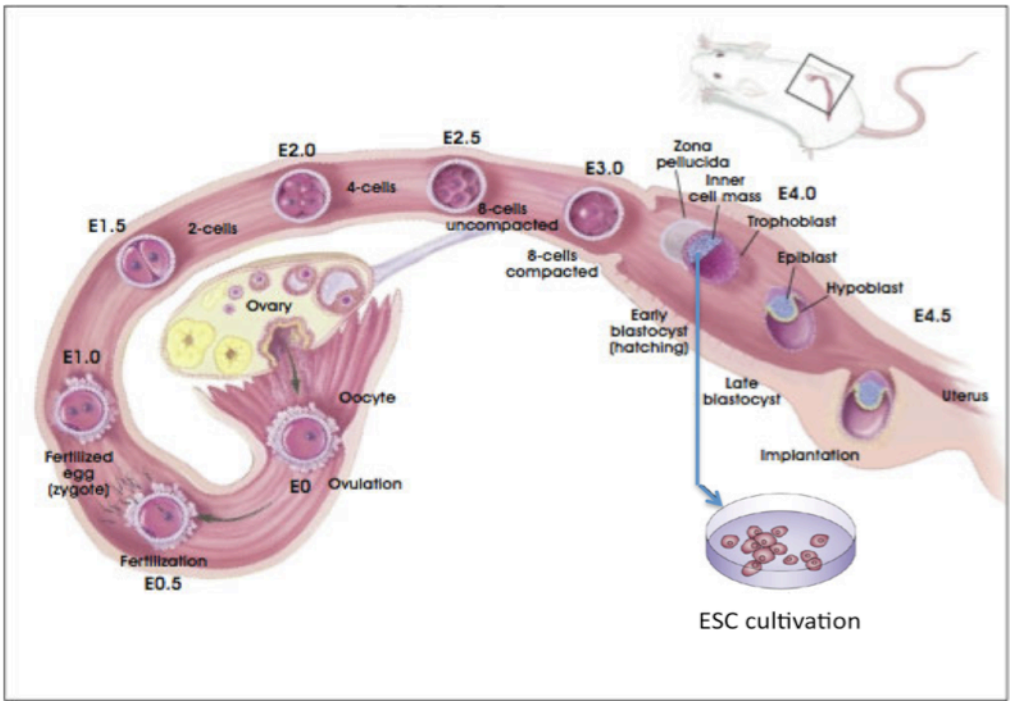


Figure 5: Mouse embryogenesis and ESC derivation. Illustration of the different stages during embryonic development in mice. ESCs are harvested from the inner cell mass and subsequently cultivated *in vitro*. Modified from http://stemcells.nih.gov/

However, alternate ways to grow ESCs have also been developed which employ inhibitors of two specific differentiation pathways - GSK3β and MEK1/2 signalling - to preserve stemness and to promote feeder-free growth. Some studies show that these culture conditions reflect the ground state of pluripotency that is found *in vivo* and are more suitable for maintenance of self-renewal of ESCs (Wray et al., 2010, Tamm et al., 2013). This has been a matter of debate in the stem cell field and the traditional method of cultivation is still being largely employed. Besides, it is not known whether blocking pathways that are important for signalling ESC differentiation can have profound effects on their replication. Hence, for the scope of this work, all experiments unless specified have been carried out under standard ESC cultivation guidelines.

A network of transcription factors function to preserve stem cell identity and regulate pluripotency by controlling gene expression of various downstream targets. Among them, Oct4, Nanog and Sox2 are the best characterized ESC markers (Loh et al., 2006, Fong et al., 2008). Oct4 expression alone is sufficient to determine the self-renewal capacity of ESCs and is markedly downregulated upon their differentiation (Pan et al., 2002). Therefore, it has been used extensively in this study to distinguish ESCs from differentiating cells.

1.3.1.3 Peculiarities of the embryonic stem cell cycle

At any given time, a typical somatic cell cycle visualized by adding a DNA stain can be illustrated as in Fig 6 - cells spend a relatively large proportion of their time in the gap phases G1 and G2 and lesser time in the S phase. On the other hand, asynchronously growing ESCs spend very little time in the gap phases and spend most of their time in the S phase (White et al., 2005). However, the actual length of S phase does not differ between other somatic cells and ESCs (Li et al., 2012). Pertinent to these observations, ESCs are often reported to have a compromised G1-S checkpoint. The tumour suppressor protein retinoblastoma (RB) that is required for prevention of aberrant G1-S progression, thereby preventing damaged DNA from being replicated, is hypophosphorylated (active) in MEFs. In contrast RB is hyperphosphorylated, rendering it inactive, in ESCs (Savatier et al., 1994). In other words, it is plausible that highly proliferative ESCs, due to the short gap phases before and after replication, may not be able to prevent damaged or partially replicated DNA from entering the subsequent replication cycle. Also in line with their high proliferative capacity, most cell cycle regulators - Cdc25a, Cdc6, cyclins etc. - are extremely abundant in ESCs compared to MEFs (Tichy et al., 2012). In addition, most of these factors - especially those required for origin licensing - remain stable throughout the cell cycle in ESCs, as opposed to being degraded and re-synthesized in a cell cycle dependant manner in other somatic cells. In contrast, the negative regulator of replication licensing, Emi1, is also maintained at high levels and is constitutively expressed in ESCs (Ballabeni et al., 2011). The factors mentioned above are known to oscillate during the cell cycle in differentiated cells, and their levels have also been reported to drastically drop down upon ESC differentiation (Fujii-Yamamoto et al., 2005). Owing to these aspects of the ESC cycle, it is tempting to speculate that replication in these cells may be intrinsically perturbed, which has been thoroughly investigated in this thesis.

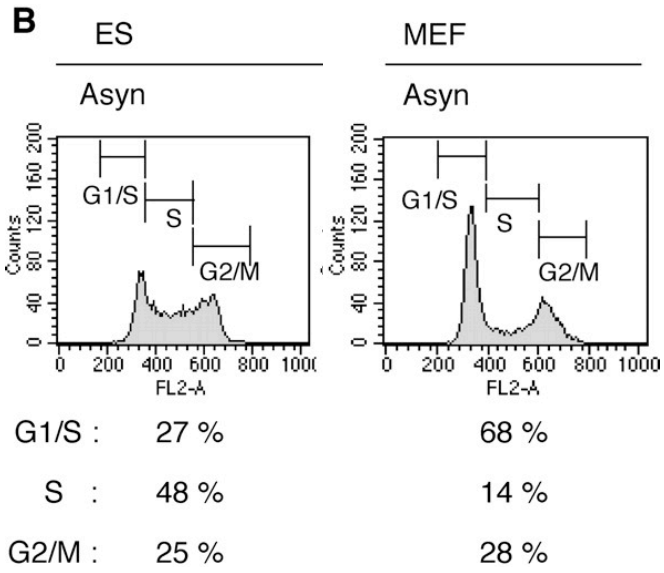


Figure 6: Differences in cell cycle profiles between ESCs and MEFs. Flow cytometry analyses of DAPI profiles reveals important differences in cell cycle distribution in ESCs and MEFs. ESCs spend most of their time in the S phase, whereas majority of MEFs are found in the G1 phase of the cell cycle. Modified from (Fujii-Yamamoto et al., 2005)

1.3.2 Induced pluripotent stem cells

Mature cells, such as fibroblasts, can be reprogrammed using the four embryonic stem cell factors - Oct4, C-Myc, Klf4 and Sox2 - into naive stem cells. The resulting cells are termed induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006). Subsequently, various other protocols have been established to generate iPSCs by using a different combination or fewer pluripotency factors (Yu et al., 2007, Shi et al., 2008, Wernig et al., 2008, Huangfu et al., 2008). Oct4 alone has been demonstrated to be sufficient for iPSC generation (Kim et al., 2009b). Alternatively, cells can also be reprogrammed by somatic cell nuclear transfer (Tachibana et al., 2013) or cell fusion experiments (Cowan et al., 2005). Important technological advances have also been made that surpass the use of viral vectors in somatic cell reprogramming (Kaji et al., 2009, Kim et al., 2009a).

iPSCs hold great potential for regenerative medicine. In principle a patient's own somatic cells can be used and successfully reprogrammed into iPSCs, which can be used for therapy. This circumvents the problems of tissue rejection and donor incompatibility that is often faced in clinics while treating many diseases. iPSCs bear striking resemblance to ESCs and share basic characteristics, which makes it easier to study stem cell biology since the rigorous

procedures involving stem cell isolation and ethical issues posed by human ESCs can be surpassed. However, there are concerns regarding their application since they exhibit DDR activation similar to cancer cells (see 1.3.4).

1.3.3 Haematopoietic stem cells

HSCs are probably the best characterized somatic stem cells. During mouse embryogenesis, HSCs arise from haemogenic endothelium and enter the foetal liver through circulation where they expand and mature (Yoshimoto and Yoder, 2009). Cycling HSCs migrate from the liver to the bone marrow just before birth and achieve maturation within four weeks, after which they seem to acquire a dormant status. It has been thought that HSC dormancy is essential to preserve its self-renewal capacity and to avoid exhaustion (Arai et al., 2009). When components of the haematopoietic system are lost, HSCs are induced to cycle via a positive feedback loop and this brings about tissue homeostasis (Trumpp et al., 2010).

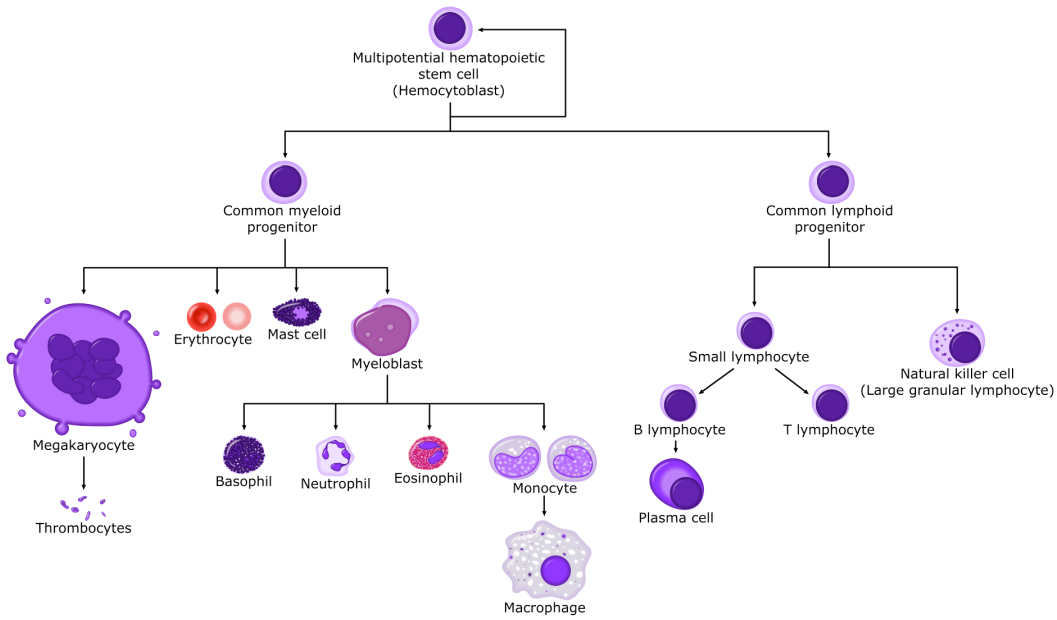


Figure 7: Haematopoiesis. Graphical representation of differentiation of HSCs into specialized cells of the haematopoietic lineage. Adopted from http://www.allthingsstemcell.com/2009/02/hematopoietic-stem-cells/

1.3.3.1 Quiescent vs. cycling HSCs

Earlier studies relying on BrdU labelling methods in combination with mathematical modelling to determine the divisional history of mouse HSCs, reveal that there are two populations of HSCs: a dormant population comprising 30% of the total HSC population, which divides every 145-193 days, and a homeostatic population that divides every 28-36 days (Wilson et al., 2008, van der Wath et al., 2009). This would imply that during the lifetime of an adult mouse, dormant HSCs divide only 5 times and are probably not responsible for regulating tissue homeostasis on a daily basis. These dormant HSCs therefore serve as a 'reserve' and are activated only upon injury or stress, whereas homeostatic HSCs are responsible for maintaining blood levels. However, BrdU itself can have an effect on HSC cycling and therefore, this proposition has been recently challenged (Takizawa and Manz, 2012). The use of carboxyfluorescein succinimidyl ester (CFSE), a fluorescent dye that is retained for upto 7-8 cell divisions and is distributed equally to daughter cells (Fig 8), coupled with mathematical simulation has revealed that there is a single cycling population of HSCs and the average HSC divides 18 times in the lifetime of a laboratory mouse. The authors suggest a dynamic model in which a certain proportion of HSCs make blood for a given time and then enter quiescence, while another lot of HSCs continues to contribute to haematopoiesis. Hence, HSCs enter quiescence and are re-activated repeatedly. This model advocates that the entire HSC pool has a similar turnover and homogenous divisional history (Takizawa et al., 2011). Furthermore, this is consistent with data from aging human HSCs, where linear telomere shortening is observed (Rufer et al., 1999).

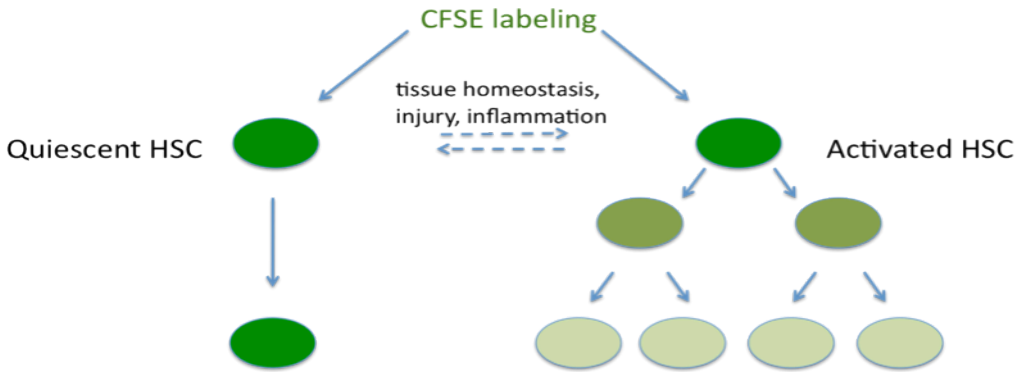


Figure 8: CFSE label retention and activation of dormant HSCs. Quiescent HSCs retain CFSE due to their dormant status, whereas activated HSCs lose the label with each round of cell division. This technique is employed to distinguish between quiescent and cycling HSCs. Quiescent HSCs can be activated upon inflammation, injury or stress. Cycling HSCs go back to dormancy upon achieving tissue homeostasis.

1.3.3.2 HSC activation

Dormant HSCs are activated into cycling in response to injury or stress (Fig 8) - for instance, during severe blood loss or upon irradiation. HSCs are also known to be activated by applying 5-florouracil, a chemotherapeutic agent (Wilson et al., 2008). As mentioned above, sustained treatment with the nucleotide analogue BrdU can also push dormant cells into proliferation (Passegué et al., 2005). However, the mechanism of activation of HSCs via these stimuli is not completely understood. Intriguingly, recent evidence shows that dormant HSCs are in fact largely resistant to chemotherapy or radiotherapy and are activated only upon stimulation by cytokines, which sensitize them to therapy. Both granulocyte-colony stimulating factor and interferon-alpha (IFN-α) induce quiescent HSCs into cycling (Morrison et al., 1997, Essers et al., 2009). Between the two cytokines, IFN-α has been shown to directly trigger HSC signalling without mobilizing it into blood and is a preferred means of HSC activation. An alternative way of activating HSCs is by lipopolysaccharide (LPS) injection, which mimics gram-negative bacterial infection (Takizawa et al., 2011). However, whether HSC induction is a direct or indirect consequence of LPS stimulation is currently under investigation (Takizawa et al., manuscript under preparation).

1.3.4 DNA damage response in different stem cell populations and consequences for aging and cancer

Our cells are subject to constant insults from either exogenous agents such as UV, chemical mutagens, smoke etc. or from various internal cellular processes. These factors can give rise to genetic alterations and every cell has a certain mutational threshold, beyond which important physiological functions might be affected. Various DNA repair mechanisms evolved to keep mutation frequency in check. Moreover, such pathways must be particularly robust in stem cells, since these are the master cells responsible for giving rise to other cells and tissues. Therefore, an active DDR is stem cells is essential to protect from growth defects or developmental abnormalities. The DDR can vary between ESCs and ASCs owing to difference in their proliferative capacity and the effect of the microenvironment on these populations. So far, the DDR in ESCs has not been extensively characterized and the available reports are largely controversial. On the contrary, DDR in ASCs has been studied comprehensively, with HSCs being the most revisited among the different ASC populations.

The two main sensors of DDR are the PI3K related kinases, ATM and ATR. The ATM pathway is activated in response to DNA DSBs whereas ATR senses long stretches of ssDNA or stalled forks when cells experience RS (for more information, see 1.2.1). If DSBs occur during G0/G1 phase of the cell cycle, when a homologous template DNA is unavailable, ATM signals effectors belonging to the non-homologous end-joining (NHEJ) pathway. This repair pathway is error prone since some information might be lost due to religation of broken ends (Fig 9). When a sister chromatid is available, i.e., during S/G2 phases of the cell cycle DSBs are preferably repaired via HR, although NHEJ also competes for repair of chromosomal breaks. Between the two pathways, HR is more efficient since it relies on repairing breaks using information from an identical, intact DNA copy.

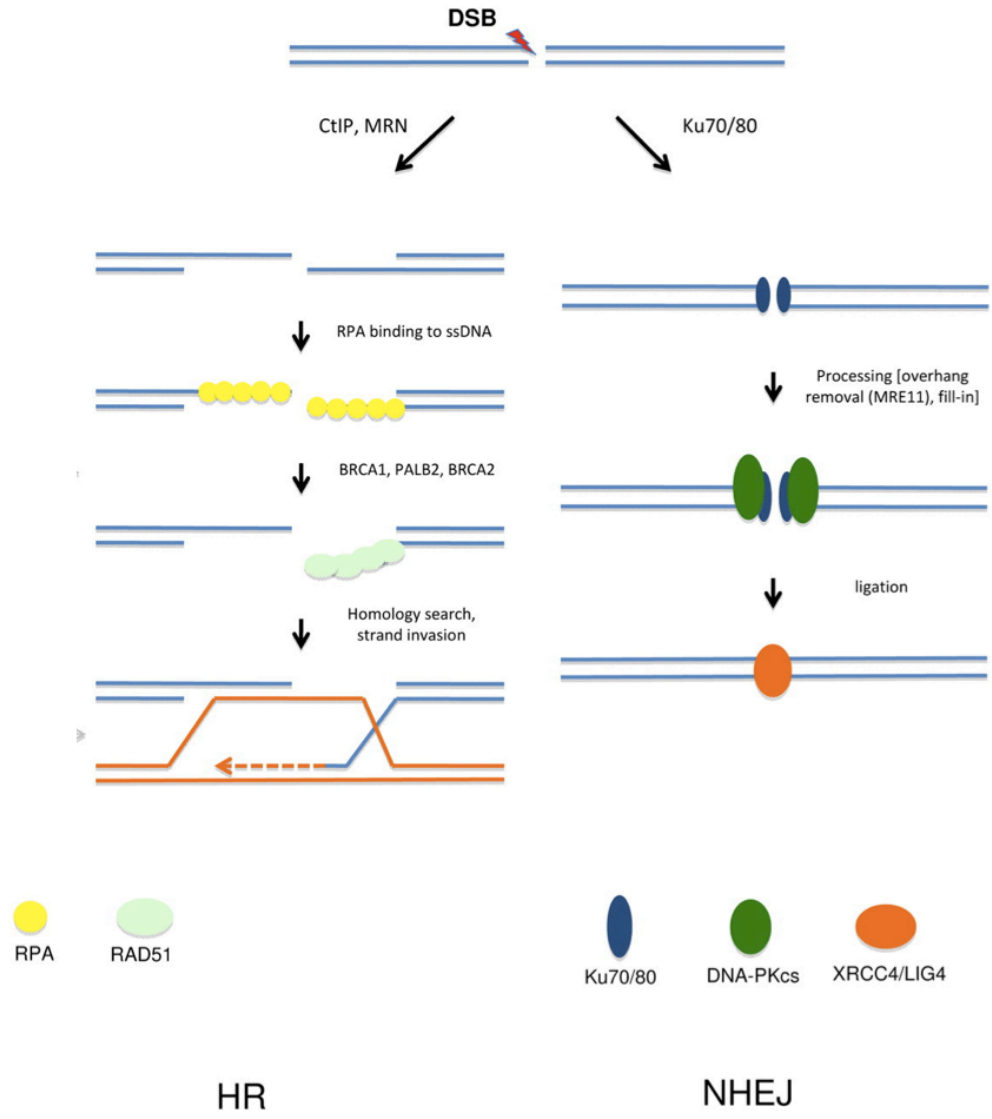


Figure 9: Repair of DSBs via HR and NHEJ. DSBs are repaired either by homologous recombination (HR), which requires an intact sister chromatid, or by non homologous end joining (NHEJ), which involves processing and re-ligation of the broken DNA ends. Modified from (Kee and D'Andrea, 2010)

ESCs have previously been reported to exhibit increased HR activity, which facilitates gene targeting in mice (Te Riele et al., 1992). A possible explanation of this hyper-recombinogenic phenotype is the functional suppression of p53 in ESCs (Aladjem et al., 1998). p53 is an essential tumour suppressor gene and is a well-known suppressor of HR (Mekeel et al., 1997). Therefore, the increased HR capacity of ESCs can be attributed to p53 suppression. Alternatively, a landmark study shows that p53 inhibits transcription of Nanog, a transcription factor required for pluripotency, and thereby promotes differentiation (Lin et al., 2005). Hence, p53 might be suppressed in ESCs to maintain stemness. Along this line, ESCs have been shown to be hypersensitive to DNA damage and undergo apoptosis or differentiation to get rid of damaged cells or to protect their genome (Van Sloun et al., 1999, de Waard et al., 2003). It has also been suggested that p53 suppression may be important to prevent cell cycle arrest during embryogenesis where rapid cell division is mandatory and increased HR could be required for timely restart of stalled replication forks (Shrivastav et al., 2008). However, evidence from other labs shows that the increased Rad51 levels in ESCs serve either to prevent illegitimate HR that is independent of p53 status or to protect stalled forks (Domínguez-Bendala et al., 2003, Tichy et al., 2012). Although ESCs exhibit about 15 times higher Rad51 protein levels compared to MEFs, the mRNA levels are only 2 times higher in ESCs and very little protein is recruited to stalled forks or during HR. In addition, despite the huge difference in Rad51 abundance, the authors observe no difference in HR efficiency between ESCs and MEFs (Tichy et al., 2012). The possible role of Rad51 during ESC replication will be discussed in this thesis.

Indeed, the p53 axis has also been exploited in reprogramming of mature cells into iPSCs. iPSC generation using the Yamanaka factors is an inefficient process and depending on the cell type, the reprogramming efficiency can drop below 1%. In 2009, a string of high profile publications reported that p53 acts as a barrier for somatic cell reprogramming (Hong et al., 2009, Kawamura et al., 2009, Li et al., 2009, Marión et al., 2009, Utikal et al., 2009). In a nutshell, reducing p53 levels in MEFs during reprogramming increases the chances of transforming somatic cells into pluripotent ones. These studies collectively proposed that inhibiting p53 limits senescence or evades apoptosis in cells, therefore increasing iPSC production efficiency. This would imply that sub-optimal cells eventually become iPSCs and hence, the genomic integrity of pluripotent cells derived using this process would be questionable. However, an alternative interpretation assumes that retroviral transduction

activates p53 dependant apoptosis and only those cells that harbour DNA damage are eliminated during reprogramming (Tapia and Schöler, 2010).

Several groups have noticed endogenous γH2AX foci formation (see 1.2.1.2) in WT ESCs and iPSCs (Saretzki et al., 2008, Banath et al., 2009, Ziegler-Birling et al., 2009, Momcilovic et al., 2010, Turinetto et al., 2012, Marión et al., 2009), but not all reports specifically comment on this observation. Those studies that have investigated the cause of γH2AX in ESCs suspect chromatin remodelling or alternative structure to be its source (Banath et al., 2009, Ziegler-Birling et al., 2009). However, there is lack of experimental evidence to support this claim. Part of the assumption stems from confusing γH2AX as an unambiguous DSB marker. As discussed in 1.2.1.2, γH2AX is also formed during RS, which can be largely uncoupled from DSB formation (Löbrich et al., 2010). For instance, Ziegler-Birling et al. report that γH2AX in ESCs does not co-localize with 53BP1, a more specific DNA DSB marker, and incorrectly conclude that γH2AX is therefore not a sign of DDR. The only legitimate conclusion from the experiment is that γH2AX is not due to DSB formation in ESCs. A recent study shows that γH2AX levels are evidently higher in ESCs compared to MEFs and decrease upon ESC differentiation. The authors also implicate γH2AX in self-renewal, but once again draw this conclusion due to the lack of 53BP1 or pATM staining in ESCs that is observed only upon DSB formation (Turinetto et al., 2012). There is perhaps just one study on presence of 'non-induced' ssDNA visualized by alkaline comet assay, thereby linking γH2AX to ssDNA breaks in ESCs (Chuykin et al., 2008). It suggests incomplete replication fork maturation in ESCs due to a faster cell cycle, but lacks mechanistic insight into how the breaks may arise. In contrast, other authors suggest that these breaks may be induced due to the susceptibility of the chromatin state in ESCs to osmotic shock experienced during the course of the experiment (Banath et al., 2009). However, what is clear from two independent studies (Ziegler-Birling et al., 2009, Turinetto et al., 2012) is that γH2AX is not due to ESC culture conditions since the inner cell mass of the blastocyst also stains positive for γH2AX. These findings *in vivo* are particularly important for this thesis.

DDR in ASCs, in contrast to ESCs, seems to be regulated quite differently. First, ASCs activate pro-survival pathways and are therefore resistant to damage induced apoptosis and senescence (Lane and Scadden, 2010, Blanpain et al., 2011). As opposed to ESCs, irradiation of ASCs - HSCs and hair follicle bulge stem cells - neither triggers p53 nor induces apoptosis. Instead, DNA damage in ASCs elicits repair and preservation of self-renewal coupled with

symmetric cell division. This enables ASCs to expand and regenerate damaged tissue. This response has been found to be dependant on p21 expression and its ability to suppress p53 (Insinga et al., 2013). Although most ASCs are quiescent and the DDR ensures their long-term survival, this comes with a cost. DDR is critical in preserving ASC self-renewal (Sotiropoulou et al., 2010), but the capacity to repair damage reduces with physiological aging and causes decline in stem cell function (Nijnik et al., 2007). ASCs enter G1 upon DNA damage, where NHEJ is the only choice available to repair DSBs. Since NHEJ is error prone, ASCs tend to accumulate mutations over time and this may lead to aging, decreased self-renewal capacity and stem cell exhaustion (Rossi et al., 2007, Mohrin et al., 2010).

Deletion of several repair factors leads to stem cell depletion and accelerates aging in mice (Park and Gerson, 2005, Sharpless and DePinho, 2007). Knocking down p53 in at least some of these mouse models rescues premature aging, but promotes tumour formation (Sahin and DePinho, 2010). It has been suggested that replicative stress during embryonic development can cause the progeroid phenotype in ATR seckel mice, which is exacerbated upon p53 deletion (Fernandez-Capetillo, 2010). Conditional knockdown of ATR (Fig 10) has also been shown to accelerate aging and leads to loss of stem cells (Ruzankina et al., 2007). In summary, evidence collectively shows that the DDR in stem cells can have relatively different outcomes. It may depend on the source of damage, the proliferative state of the stem cell or the developmental stage of the animal.

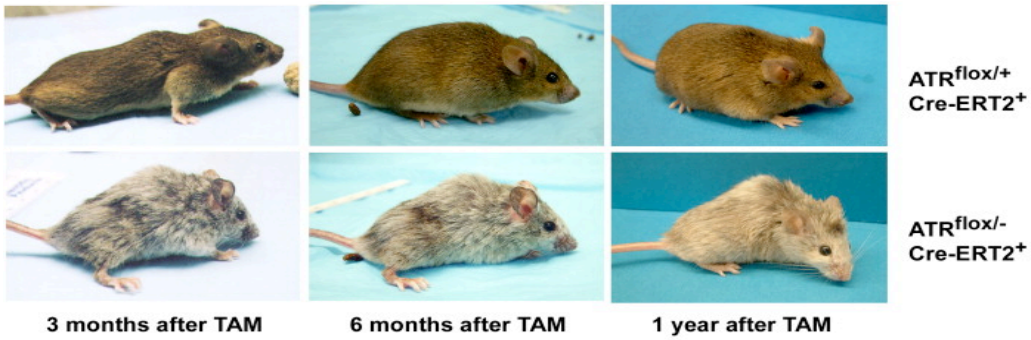


Figure 10: Accelerated aging in ATR deficient mice. WT mice harbouring intact ATR ($ATR^{flox/+}$) age normally, while conditionally knocking down ATR ($ATR^{flox/-}$) by injecting tamoxifen (TAM) accelerates aging as observed by greying of hair and kyphosis. Adopted from (Ruzankina et al., 2007).

1.4 Epigenetic mechanisms that may be coupled to DDR in ESCs

Epigenetics is defined as a set of heritable changes that do not alter the DNA sequence, but influence gene expression. Examples of epigenetic mechanisms include DNA methylation and demethylation, histone modifications and regulation of gene expression by non-coding RNA. There is enough evidence that epigenetic modifications can widely contribute to DDR in a variety of cell types (Hoeijmakers, 2009). Several reports highlight the role of (de)methylation in reprogramming during early embryonic development (Meissner et al., 2008, Wossidlo et al., 2010, Hajkova et al., 2010, Bhutani et al., 2010, Popp et al., 2010, Cortellino et al., 2011, Smith et al., 2012), hence special emphasis will be laid on these aspects in this chapter.

1.4.1 Active vs. passive demethylation

The most common form of DNA methylation is the addition of a methyl group on the fifth carbon of Cytosine (5-methylcytosine or simply 5mC) by DNA methyltransferases (Fig 10). DNA methylation in ESCs occurs mainly in non-CpG islands (Ramsahoye et al., 2000) and is responsible for repression of gene function. This is followed by DNA demethylation, which may occur actively or passively, and involves removal of modified bases to mark the onset of gene expression. It has been proposed that active demethylation can occur at tissue specific promoters during early embryogenesis (Shemer et al., 1991). However, demethylation has also been shown to occur passively via chromosomal replication during early embryonic development (Rougier et al., 1998).

1.4.2 Role of base excision repair (BER) factors in active demethylation

A number of recent studies demonstrate that demethylation is, at least partially, an active mechanism in mouse ESCs. 5mC can be oxidized by the family of ten-eleven translocation factors (TET1/2/3), which are dioxygenases. The TET family of proteins is also important in maintaining pluripotency (Bhutani et al., 2011, Wu et al., 2011, Costa et al., 2013). The product of 5mC oxidation is 5-hydroxymethylcytosine (5hmC) (Tahiliani et al., 2009, He et al., 2011). 5hmC can be further oxidized by the TET family to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (Ito et al., 2010). 5fC and 5caC are actively removed at distal

elements to promote pluripotent gene expression in ESCs by thymine DNA glycosylase (TDG) (He et al., 2011, Shen et al., 2013, Song et al., 2013) (Fig 11). TDG is an enzyme that belongs to the BER pathway. The BER pathway removes modified/damaged bases via DNA glycosylases, which helps prevent mispairing and hence erroneous DNA replication. TDG was discovered in the early nineties and was shown to specialize in removal of G-T mispairs that form as a result of spontaneous deamination of 5mC (Wiebauer and Jiricny, 1990). The enzyme apurinic/apyrimidinic endonuclease 1 (APE1) cleaves the sugar phosphate backbone 5' of the AP site to prime DNA synthesis (Mol et al., 2000), thereby creating an apyrimidinic (AP) site. This allows for TDG to excise the mispaired thymine, or 5fC/5caC for that matter. Therefore, it is plausible that active demethylation elicits a DDR since modified bases are evicted and this may result in strand breaks (Wossidlo et al., 2010).

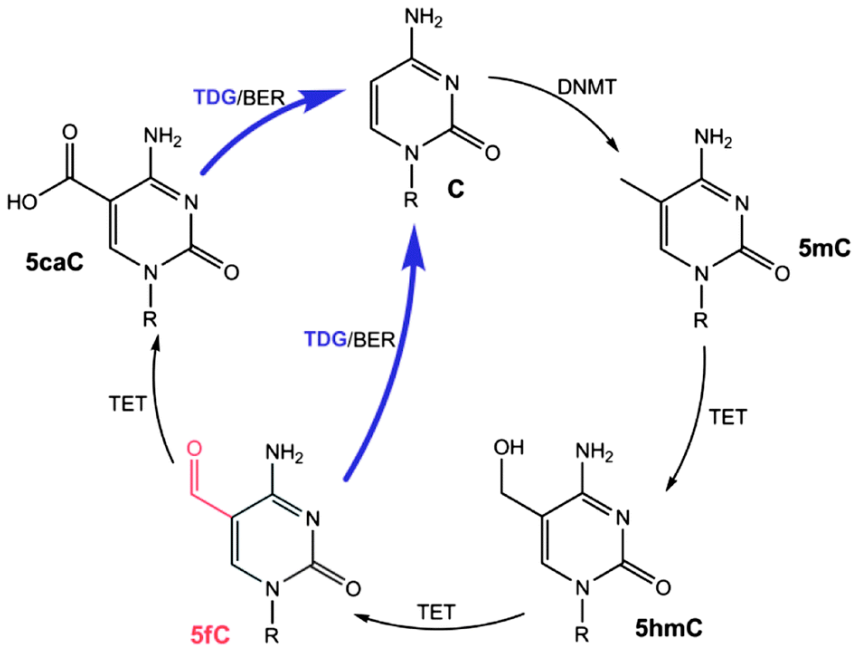


Figure 11: Role of BER in active demethylation. Cytosine (C) can be converted to 5-methylcytosine (5mC) by the enzyme DNA methyltransferase (DNMT), which can be further oxidized to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) by the dioxygenase ten-eleven translocation factor (TET). 5fc and 5caC are substrates for thymine DNA glycosylase (TDG) or other factors belonging to the base excision repair (BER) pathway, which actively demethylate these substrates back to C. Adopted from (Xu et al., 2014).

1.4.3 Other repair mechanisms implicated in demethylation

It has been proposed that the nucleotide excision repair (NER) pathway can also play a role in active demethylation (Niehrs and Schafer, 2012). NER helps in the removal of bulky DNA adducts, especially those that may be induced by UV. 5mC has been shown to be a direct substrate of growth arrest and DNA damage inducible protein protein 45 alpha (Gadd45a), which recruits the NER machinery via its interaction with xeroderma pigmentosum complementation group G (XPG) protein (Barreto et al., 2007). Gadd45a is a sensor that is activated upon DNA damage and modulates repair pathways in response to cellular stress. However, the mechanism by which Gadd45a assists NER during demethylation is unclear and even the role of Gadd45a in demethylation itself has been controversial (Jin et al., 2008).

1.5 Diverse cellular roles of PARP1

PARP1 enzyme is involved in diverse cellular processes (Fig 12). As the name suggests, its main role is to synthesise poly ADP ribose polymers (Okayama et al., 1977). It contains two zinc finger domains that are required for its binding to single and double strand DNA breaks (Gradwohl, 1990). A third zinc finger domain helps in modulating the catalytic activity of PARP1 upon DNA damage induction (Langelier et al., 2008). Both NAD+ and ATP are essential for PARP1 activity and even more so upon DNA damage (Berger et al., 1986, Carson et al., 1988). PARP1 poly(ADP) ribosylates numerous targets including itself (Altmeyer et al., 2009, Tao et al., 2009). Within its self-modification domain, it also includes a BRCA1 carboxy terminal (BRCT) repeat motif that is found in other players involved in DDR (25) (Kameshita et al., 1984). Certain types of tumours are deficient in BRCA and rely on PARP1 activity for survival. This aspect has been thoroughly investigated for chemotherapeutic purposes (Rouleau et al., 2010) and Olaparib, a specific PARP1 inhibitor (Fong, 2009), is currently in phase III clinical trials.

1.5.1 Base excision repair

PARP1 is an important factor in the BER pathway and interacts with XRCC1 via its BRCT domain to mediate repair (El-Khamisy et al., 2003). In light of the interplay between BER and epigenetics (see 1.4.2), PARP1 has also been implicated in DNA demethylation (Hajkova et al., 2010, Wossidlo et al., 2010).

1.5.2 Chromatin structure, transcription and pluripotency

Chromatin structure can influence transcription by limiting RNA polymerase II loading or initiation/elongation of transcription (Li et al., 2007). The linker histone H1 can modulate chromatin structure to regulate transcription (Happel and Doenecke, 2009). PARP1 competes with H1 for nucleosome binding and in most cases, the promoter occupancy is reciprocal (Krishnakumar et al., 2008). Depletion of PARP1 can cause gross alterations in chromatin structure (Tulin and Spradling, 2003). PARP1 has also been shown to regulate histone modifications such as acetylation and methylation to regulate transcription. PARP1 also acts as a transcriptional regulator for some genes irrespective of its ability to modify chromatin. It has been shown to be required for retinoic acid (RA) induced transcription, independent of its catalytic activity (Pavri et al., 2005).

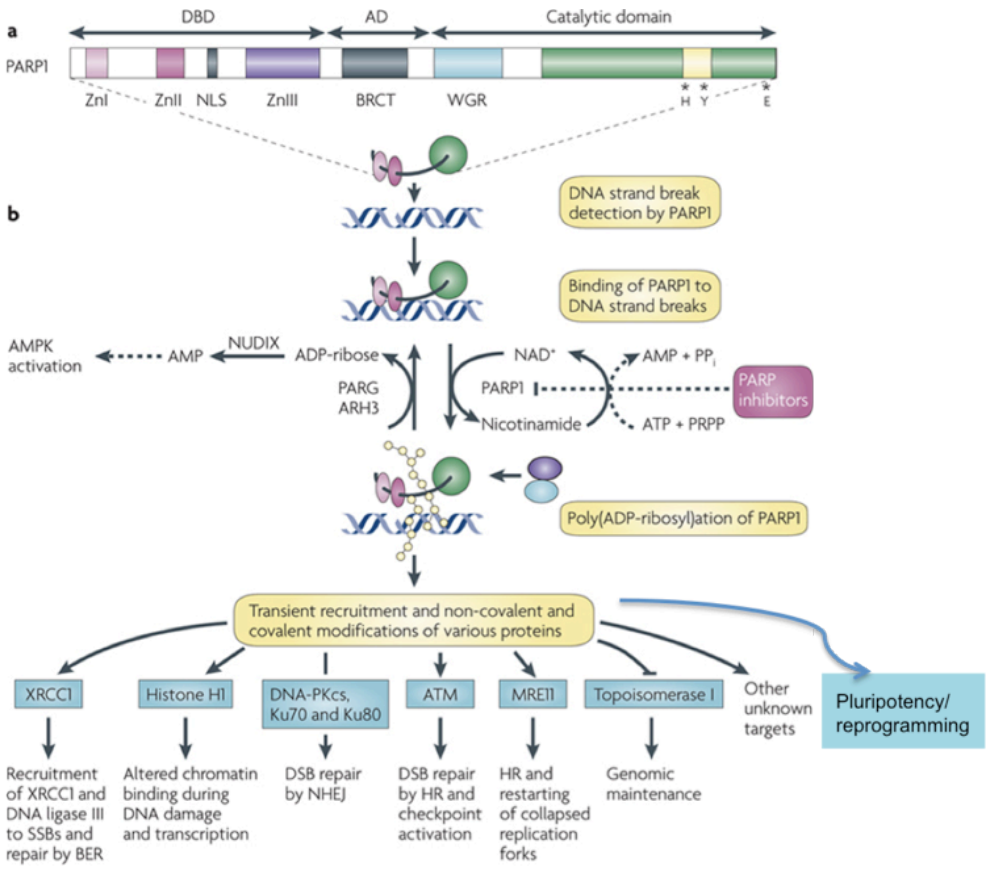


Figure 12: Diverse cellular roles of PARP1. PARP1 consists of a DNA binding domain (DBD), an activation domain (AD) and a catalytic domain. It is capable of detecting DNA strand breaks and binding to DNA. It brings about poly(ADP) ribosylation of various factors including itself and is therefore involved in a variety of processes such as base excision repair (BER), chromatin remodelling, transcription, pluripotency and genome maintenance. Modified from (Rouleau et al., 2010).

The control of pluripotency is governed by both chromatin structure and transcription. ESCs have been shown to possess higher order chromatin structure and active chromatin that permits transcription of stemness associated genes (Meshorer and Misteli, 2006). PARP1 is also required for establishment of pluripotency and has been shown to regulate reprogramming of mature cells into stem cells by early stage epigenetic modifications (Doege et al., 2012) and via its interaction with Sox2 (Weber et al., 2013).

1.5.3 Fork protection

A crucial finding from a recent study in our lab demonstrates the role of PARP1 in replication fork protection independent from its BER function. Under conditions of drug induced replication stress, PARP1 inhibition leads to chromosomal breakage (Chaudhuri et al., 2012). Subsequently, it has been shown that PARP1 PARylates the helicase RecQ1, which is required for restart of stalled replication forks, and prevents untimely restart of reversed forks that are formed upon Top1 inhibition. In agreement with this, upon treatment with CPT and in the absence of PARP1 activity, RecQ1 promotes premature fork restart, leading to replication forks collapse into DSBs (Berti et al., 2013).

Therefore, PARP1 activity is expected to be required in any condition leading to replication of a damaged or discontinuous template, and is of particular relevance for this study.

2. SPECIFIC AIMS

During ESC division, the gap phases are very short but the S-phase length is unaffected- indicating that ESCs spend most of their time in the S phase. The quick transition between G1-S phase also correlates with increased amounts of replication licensing factors (Fujii-Yamamoto et al., 2005). Replication origin distribution varies between ESCs and differentiated cells and origin density in ESCs is higher than committed cells (Hiratani et al., 2008). However, in a system where DNA replication is paramount, little is known about how ESCs cope with the need to replicate quickly and efficiently. Hence, the main aim of this thesis is to investigate whether ESCs experience endogenous replication stress, and to elucidate the underlying mechanism.

HSCs are multipotent and give rise to all other haematopoietic cells. In contrast to ESCs, adult HSCs are largely quiescent and are activated in response to injury, stress or inflammation and bring about tissue homeostasis. Along the same line, it is largely unknown how HSCs transit from G0 to G1/S and regulate DNA replication upon activation. It is tempting to speculate that HSCs may experience replication stress upon exit from dormancy. Therefore, this study also includes pilot experiments to start understanding how HSCs cope with the sudden need to replicate, which entails their activation.

3. MAIN RESULTS

3.1 Endogenous replication stress in ESCs

3.1.1 High basal levels of γH2AX in ESCs compared to differentiating cells

To investigate DDR in unperturbed ESCs, we performed immunofluorescence (IF)-based stainings for various markers. As previously reported (Banath et al., 2009, Turinetto et al., 2012), ESCs exhibit elevated levels of endogenous γH2AX, which correlates with the pluripotency marker Oct4 (Fig 13, upper panel). Upon induction of differentiation, cells progressively lose Oct4 and those cells that lose Oct4 do not exhibit γH2AX (Fig 13, lower panel). These results were confirmed in another ESC line, JM8 (Fig 14). To check whether the loss in γH2AX in differentiating cells was because of reduced proliferative capacity, EdU/DAPI content was assessed by flow cytometry. There is no apparent difference in the percentage of proliferating cells between ESCs and differentiating cells (ESCs grown for 5 days without LIF) at this time point (Fig 15).

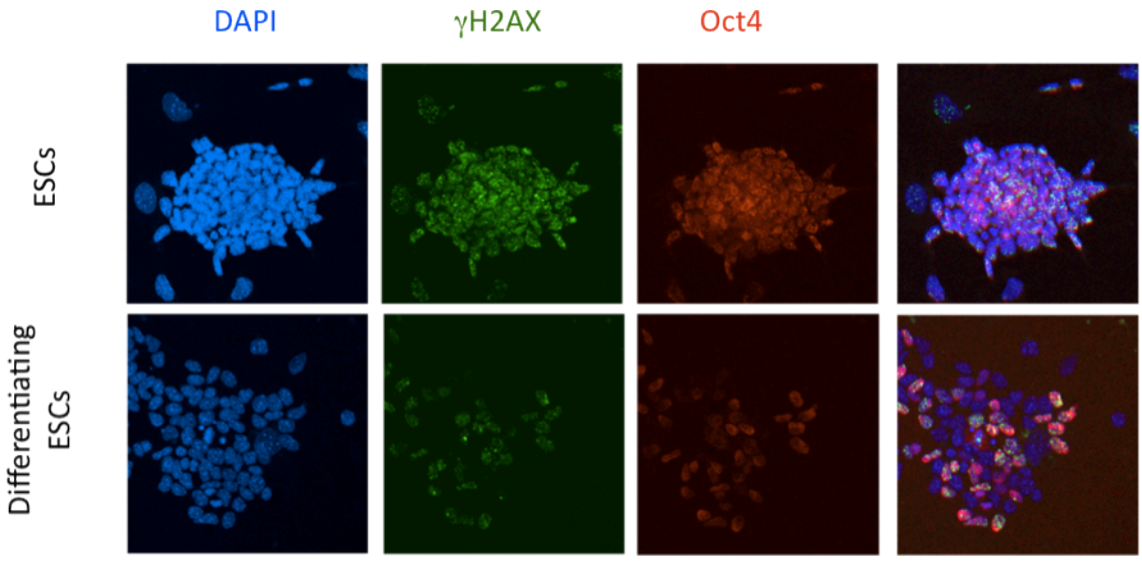


Figure 13: High basal levels of γH2AX in ESCs. Pluripotent ESCs that stain positive for Oct4 harbor high levels of γH2AX (upper panel). Upon differentiation (ESCs cultivated in the absence of LIF for 5 days), cells lose Oct4 and γH2AX simultaneously (lower panel). This experiment was repeated several times, with highly reproducible results.

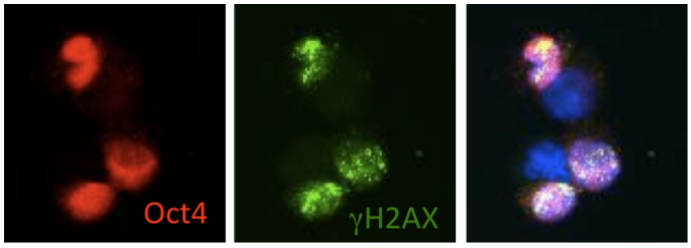


Figure 14: DDR staining in cultured JM8 ESCs upon partial differentiation. JM8 cells induced to differentiate by cultivation in media without LIF. The representative image is taken 5 days after LIF removal and shows that cells that have lost Oct4 at this time point of differentiation have also lost the DDR marker γH2AX. The experiment has been reproduced twice, showing similar results.

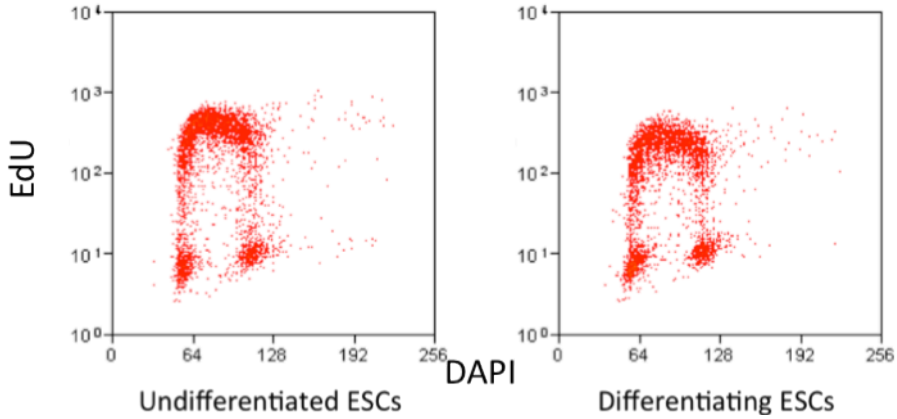


Figure 15: Reduction in γH2AX levels in differentiating cells is not due to decrease in proliferative capacity. Percentage of proliferating cells monitored by flow cytometry, as EdU positive cells. Differentiating ESCs were cultivated in the absence of LIF for 5 days. The fraction of EdU-positive cells at this time point of differentiation is comparable to that observed in undifferentiated ESCs. EdU is a nucleotide analogue and DAPI a DNA dye.

3.1.2 Co-localization of γH2AX with other DDR markers

γH2AX in unperturbed ESCs was previously reported, but the lack of its co-localization with a DSB marker (53BP1) led the authors to exclude its role in DNA damage signalling; and they rather interpret it as a marker of different chromatin organization (Banath et al., 2009, Ziegler-Birling et al., 2009) or associate it with self-renewal (Turinetto et al., 2012). Hence, to assess if non-challenged ESCs also exhibit other DDR markers, γH2AX and Oct4 were co-stained with the specific DSB marker 53BP1, but also with the ssDNA binding proteins RPA and Rad51.

3.1.2.1 *In vitro*

All cultured ESCs positive for γH2AX are also positive for the ssDNA binding proteins RPA and Rad51 (Fig 16) - suggesting that these highly proliferating cells display frequent ssDNA discontinuities on their replicating chromosomes, which is a hallmark of RS (see 1.2.1.2). These results are not dependent on the specific cell line or cell cultivation method, since different ESC lines grown in alternative ESC media (2i+LIF, see materials and methods) also exhibit γH2AX, RPA and Rad51 foci (Fig 17). Upon conditions of partial differentiation, not yet interfering with cell proliferation (Fig. 15), cells that lose Oct4 and γH2AX also rapidly lose RPA and Rad51 foci (Fig 18), suggesting that the presence of ssDNA is intrinsically linked to stemness and not simply to their high proliferation rate.

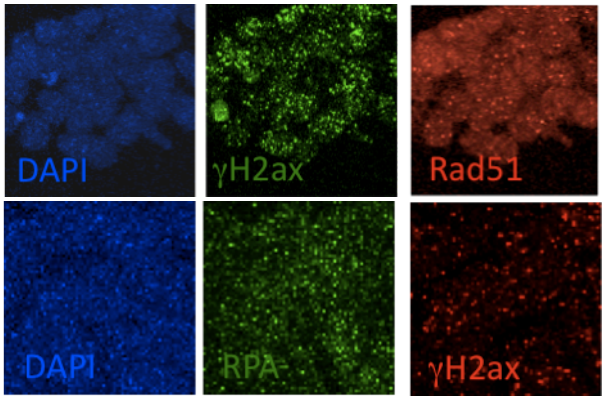


Figure 16: ESCs are co-positive for γH2AX and ssDNA binding proteins RPA and Rad51 in ESCs. IF analyses of γH2AX, RPA and Rad51 in undifferentiated ESCs.

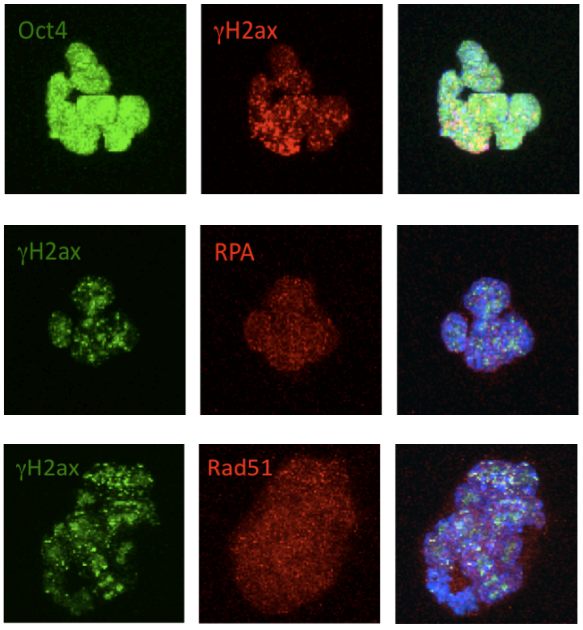


Figure 17: Higher levels of DDR markers is irrespective of cell line/culture methods. IF analyses with indicated markers in different ESC line Stat3 cultivated in the alternative stem cell media 2i+LIF.

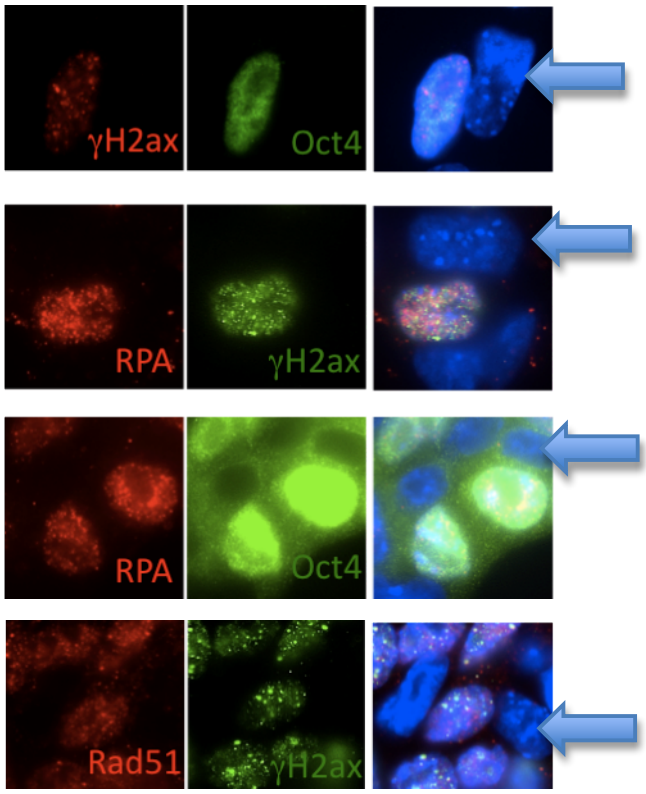


Figure 18: ESCs undergoing differentiation also lose RPA and Rad51 along with γH2AX and Oct4. IF on partially differentiating ESCs (cells grown in the absence of LIF for 5 days). Examples of differentiating cells that lose DDR markers in parallel to Oct4 are indicated with solid blue arrows.

In agreement with previous studies (Banath et al., 2009, Ziegler-Birling et al., 2009), γH2AX-positive ESCs do not exhibit 53BP1 foci, ruling out spontaneous DSB formation as the source of genotoxic stress (Fig 19). Overall, these data strongly suggest that cultured ESCs experience signs of replication stress in the absence of chromosomal breakage.

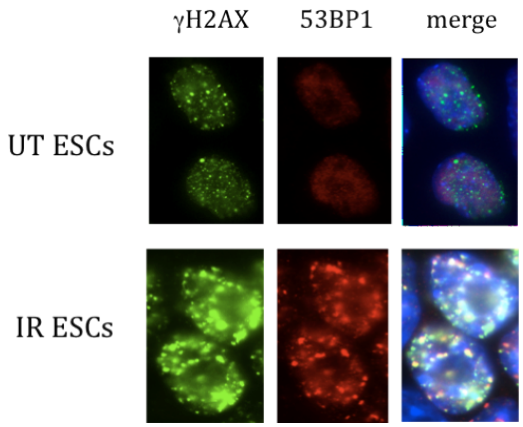


Figure 19: Endogenous γH2AX foci in ESCs are not due to DSB formation. Single cell IF studies confirm that γH2AX foci do not co-localize with 53BP1 in untreated ESCs (upper panel). DSBs are formed only upon irradiating ESCs (10 Gy), and can be visualized by clear co-localization of γH2AX foci with 53BP1 foci (lower panel).

3.1.2.2 *In vivo*

We also confirmed punctuate γH2AX foci formation within the mouse blastocyst where the ESCs reside. Further, all cells in the blastocysts also stain positive for RPA (courtesy J. Mendez), emphasizing that ssDNA accumulation underlies DDR activation in ESCs *in vivo*. These observations confidently exclude that the phenomena observed *in vitro* are due to cultivation artefacts and suggest that they rather reflect inherent stress associated with the pluripotent state of ESCs (Fig 20).

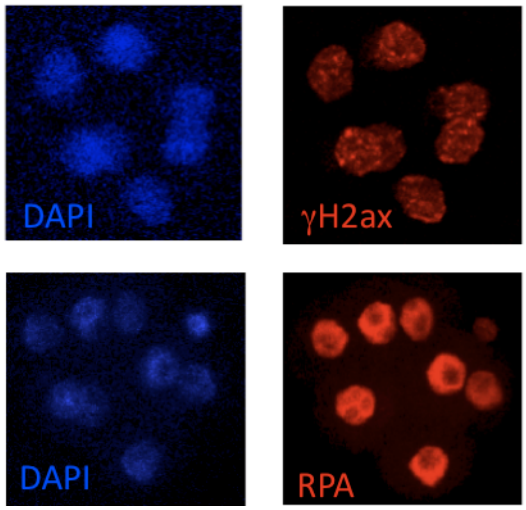


Figure 20: Blastocysts accumulate ssDNA regions. *In vivo* IF analyses for γH2AX and RPA clearly reveals that cells within the blastocyst stain positively for both these markers. Courtesy J. Mendez, CNIO, Madrid.

3.1.3 Accumulation of ssDNA gaps as visualized by transmission electron microscopy

As these data suggest the presence of ssDNA regions in these fast-replicating cells, we next visualized the fine architecture of replication intermediates (RI) by psoralen crosslinking coupled to transmission electron microscopy (EM), according to powerful protocols established in the lab (Neelsen et al., 2014). This analysis strikingly revealed that the vast majority (82%) of the replication forks in ESCs do accumulate ssDNA gaps/nicks (Fig 21). In agreement with the RPA and Rad51 staining, the percentage of replication forks with ssDNA gaps also drastically and rapidly drops down upon induction of differentiation (Fig 21, lower panel). The efficiency of differentiation in the population of cells used for RI extraction was determined by staining for Oct4 by flow cytometry (Fig 22). These results confirm that majority of the cells have undergone differentiation at this time point, and lose γH2AX in parallel, in agreement with Fig 13. The residual subpopulation of undifferentiated Oct4-

positive cells in the differentiating sample (Fig 22) may well explain the residual number of ssDNA gaps observed by EM (Fig 21, lower panel), reinforcing their correlation with the pluripotent state.

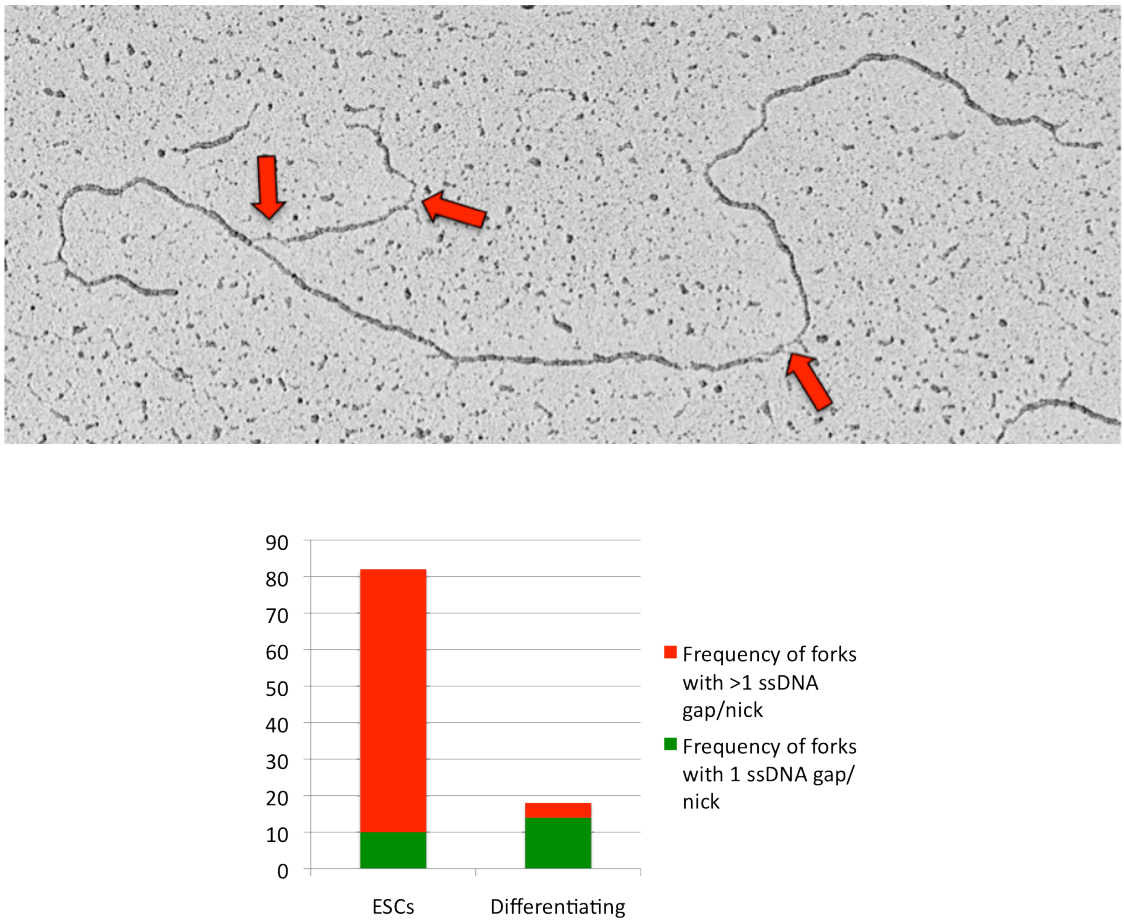


Figure 21: Replication intermediates from ESCs present numerous ssDNA gaps/nicks. Upper panel: replication intermediates (RI) in ESCs visualized by TEM. Solid red arrows indicate ssDNA gaps. Lower panel: quantification of RI presenting ssDNA gaps/nicks in ESCs and differentiating cells. Frequency of RI presenting ssDNA gaps drops down upon differentiation. This experiment has been reproduced twice.

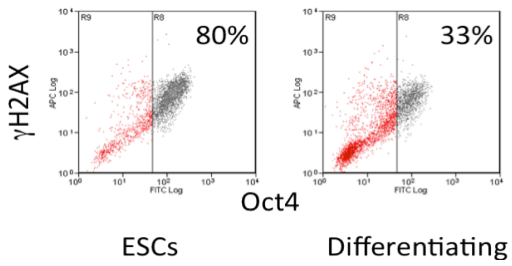


Figure 22: Quantitative analysis of differentiation. Flow cytometry analyses reveals 80% of ESCs (left panel) are positive for Oct4 and γH2AX. Most cells lose Oct4 and γH2AX upon induction of differentiation (right panel). Differentiating cells shown in red.

3.1.4 Increased fork reversal

Evidence from other studies in our lab shows that detection of ssDNA gaps is often coupled with replication fork reversal (see 1.2.3.1). Fork reversal is a conserved mechanism in higher eukaryotes and occurs frequently when replication is perturbed (Neelsen and Lopes, *in press*). In full agreement with these reports and with the evidence above on ssDNA accumulation, ESCs display an unusually high proportion (30%) of reversed replication forks and their frequency markedly and rapidly decreases upon induction of differentiation (Fig 23). once again, residual reversed forks early after induction of differentiation may reflect the minority of cells still displaying stem cell characteristics and active DDR (Fig 22).

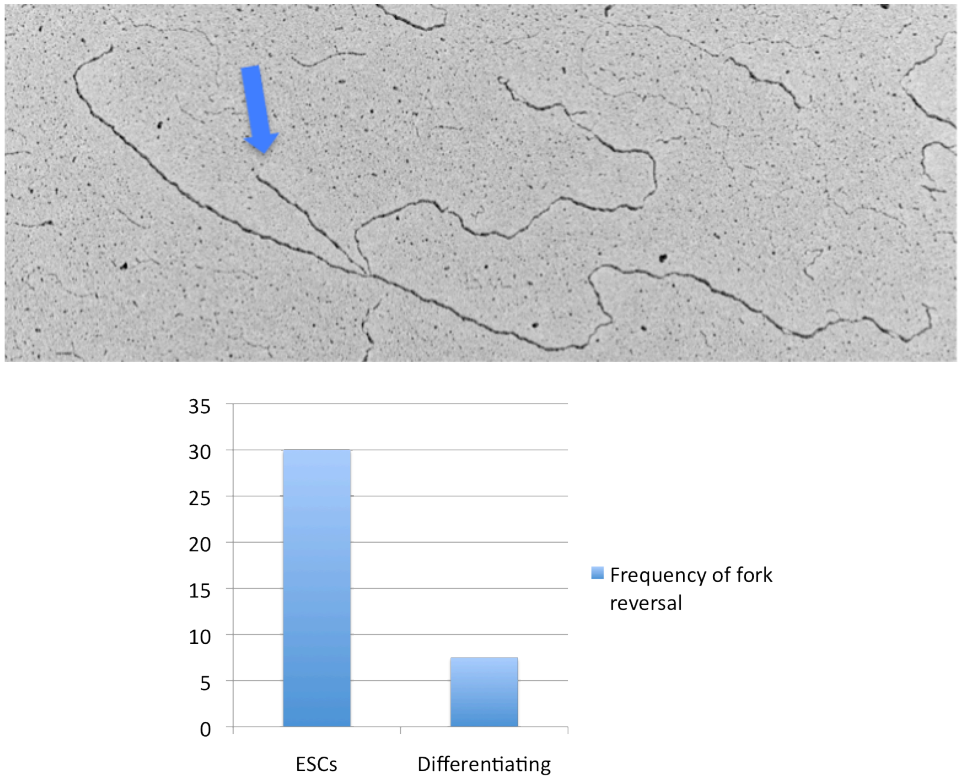


Figure 23: Increased fork reversal in unperturbed ESCs. Upper panel - replication intermediates in ESCs visualized by TEM. This is a representative picture of a reversed fork; the blue arrow indicates the regressed arm. Lower panel - frequency of replication fork reversal in ESCs and differentiating cells. This experiment has been reproduced twice.

3.1.5 Slow fork progression

From several previous studies, it is now clear that fork reversal accompanies replication fork slowdown (Chaudhuri et al., 2012, Neelsen et al., 2013a, Neelesen et al., 2013b). Considering the above evidence of RS in stem cells, we decided to directly investigate the progression of individual replication forks by "DNA fiber analysis", a method based on cellular uptake and

incorporation of halogenated nucleotides, spreading of DNA fibers on glass slides and detection of replicated tracts by specific antibodies. By successive incorporation of two different halogenated nucleotides, ongoing replication forks can be identified by the red-green pattern revealed after IF staining (for further details, see methods). This single-molecule analysis, applied statistically to a large number of tracts, shows that entire population of replication forks in ESCs travel much slower than in differentiating or differentiated cells (Fig 24).

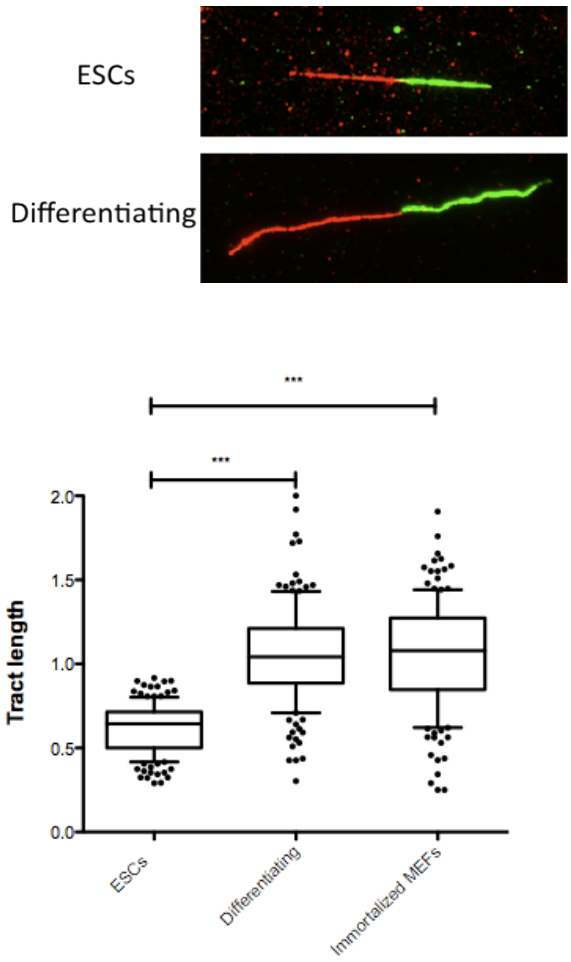


Figure 24: Replication forks in ESCs travel slower than in differentiating cells. Upper panel: representative images of DNA fibers in ESCs and differentiating cells after 20min labeling (see methods). Replication tracts in ESCs are much shorter than in differentiating cells. Lower panel: statistical analyses for differences in tract length between ESCs and differentiating/differentiated cells (Mann-Whitney test). This experiment has been reproduced several times. *** corresponds to P value < 0.0001

Taken together, all the hallmarks of RS - γH2AX, the frequency of forks with ssDNA gaps/nicks and of reversed forks, and the delayed fork progression - are quickly lost upon

onset of differentiation, before cell proliferation is detectably affected (Fig 15). These data clearly demonstrate that ESCs experience endogenous RS.

3.1.6 ATR phosphorylates H2AX in response to RS

ssDNA accumulation is typically sensed by the ATR kinase, leading to H2AX phosphorylation (Ward and Chen, 2001). Hence, we investigated whether treatment with a specific ATR inhibitor would inhibit constitutive H2AX phosphorylation in ESCs. ATR inhibition causes a significant reduction in EdU incorporation, emphasizing its importance for unhindered replication in this system (Fig 25, upper panel). Moreover, inhibiting ATR activity causes a sharp decrease in γH2AX levels, demonstrating that RS in ESCs is mainly channelled through the ATR pathway (Fig 19, lower panel). In agreement with this finding, earlier reports had shown that other PI3K related kinases (ATM, DNA-PKcs) are not responsible for H2AX phosphorylation in ESCs (Banath et al., 2009).

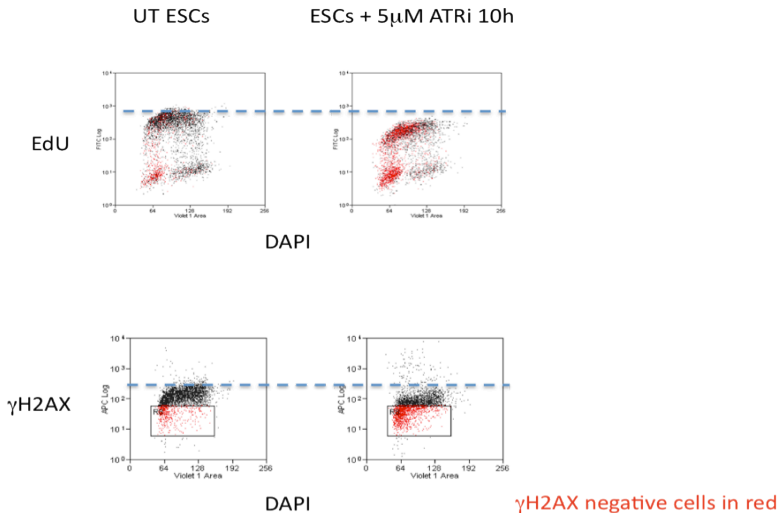


Figure 25: ATR phosphorylates H2AX in response to RS. Flow cytometry analyses after ATR inhibition reveals reduction in EdU incorporation (upper panel) and decline in γH2AX levels (lower panel). The decrease in cell proliferation and loss in H2AX phosphorylation upon ATR inhibition is highly reproducible.

3.2 Role of PARP1 in fork protection in ESCs

Poly-ADP ribose polymerase 1 (PARP1) plays important roles in various cellular processes including DNA repair and maintenance of pluripotency (see 1.5). More recently, our lab has shown that PARP1 activity is required to protect forks from breakage in conditions of drug induced RS. Camptothecin (CPT) is a specific topoisomerase 1 (Top1) inhibitor and leads to

nick accumulation on the template by trapping the Top1 complex. While treatments with Olaparib, a specific PARP1 inhibitor, has only marginal effects in somatic cells, combining mild CPT doses with Olaparib, leads to formation of DNA double strand breaks (DSBs), due to "run-off" of unprotected replication forks on the discontinuous template (Chaudhuri et al., 2012). The resulting DNA DSBs can be visualized by staining for a specific marker - i.e. 53BP1 foci - or by pulse field gel electrophoresis (PFGE), a physical method used for detecting chromosomal breakage.

We tested whether PARP1 activity could be particularly important in unperturbed ESCs, which are inherently stressed and present ssDNA gaps, thus phenocopying CPT-treated somatic cells. Treating ESCs with mild doses of Olaparib causes drastic increase in γH2AX and massive chromosomal breakage as indicated by co-localization of γH2AX with 53BP1 foci and physical DSBs detected by PFGE (Fig 26 and 27). Thus, as predicted by our model, PARP1 activity is crucial in preventing replication forks from collapsing in unperturbed ESCs. Accordingly, treatment of ESCs with low doses of CPT brings about only a modest increase in γH2AX and DSB formation, supporting the notion that the type of lesions induced by mild CPT treatments (i.e. ssDNA gaps/nicks) is endogenously present in ESCs.

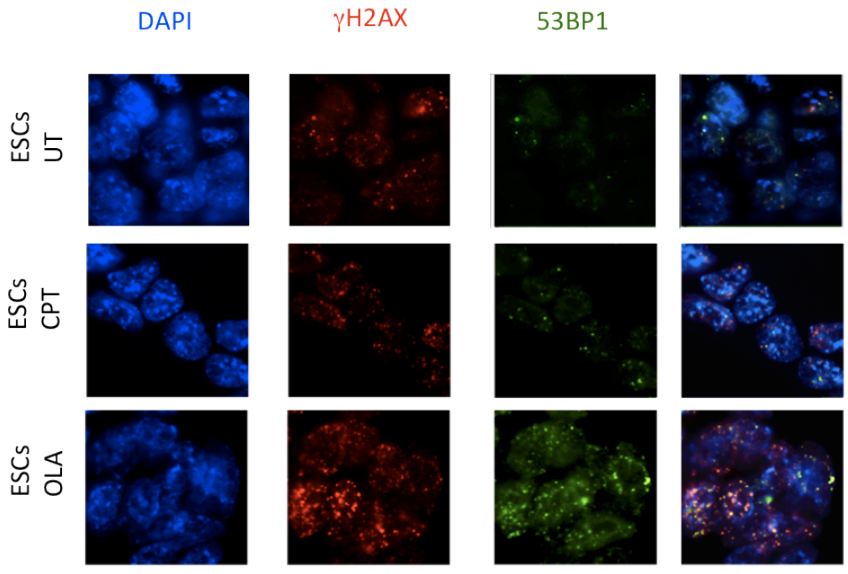


Figure 26: Inhibition of PARP1 activity causes chromosomal breakage in ESCs. PARP1 inhibition (10μM, 1h) leads to increase in γH2AX foci and DSB formation visualized by 53BP1 foci co-localization in ESCs by IF (bottom most panel). Low doses of CPT (25nM, 1h) cause a modest increase in break formation (second panel from top). Ola is short for Olaparib. This experiment has been reproduced several times.

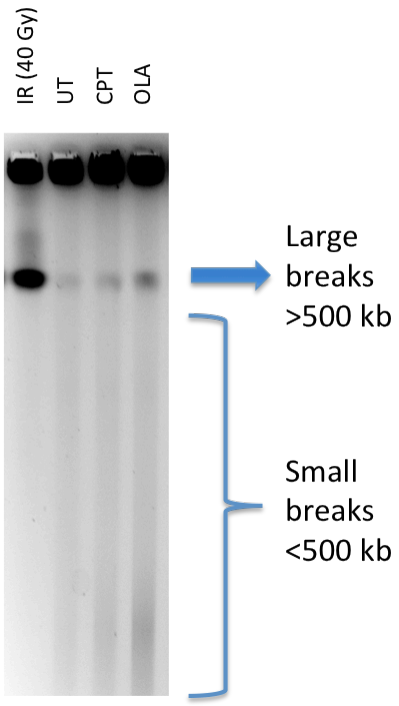


Figure 27: Inhibition of PARP1 activity causes chromosomal breakage in ESCs. DSB formation upon PARP1 inhibition (10μM, 1h) visualized by PFGE (compare lanes 2 and 4). Larger breaks (>500 kb) run as a single band on the top of the gel, indicated by solid blue arrow; smaller breaks run as a smear (<500 kb, indicated by blue flower bracket). ESCs irradiated with 40 Gy used as positive control for DSB detection. Ola is short for Olaparib. This experiment has been reproduced thrice.

3.3 Possible causes of RS in ESCs

Having established that ESCs experience endogenous RS due to ssDNA gaps and that this is accompanied by DSB formation upon PARP inhibition, we investigated possible sources/causes of RS (see 1.2.2). γH2AX was hereafter used as a marker for ssDNA gaps owing to reliable staining quality and available established protocols to follow reduction/increase in levels, either by single-cell IF pattern of γH2AX foci or by FACS based quantification of γH2AX staining. Any treatment that exacerbates RS will lead to an increase in γH2AX (as in the case of PARP1 inhibition), while removal of the plausible source of RS would be expected to cause reduction in γH2AX levels. If γH2AX foci pattern and overall intensity remain unchanged, this implies that the tested perturbation does not have any impact on the generation of endogenous RS in ESCs.

3.3.1 Oxidative DNA damage

Oxidative DNA damage is the most common source of RS in cells caused by spontaneous production of free radicals as byproducts of various cellular processes (see 1.2.2.6). We tested whether presence of excessive free radicals was the source of RS in ESCs. N-acetylcysteine (NAC) and β-mercaptoethanol (β-ME) are potent scavengers of free radicals. It should be noted that 0.1mM β-ME is a typical supplement in ESC culture. Exposing ESCs to prolonged

treatments with both reducing agents had no visible effect on γH2AX signalling in ESCs (Fig 28), indicating that oxidative DNA damage is not a major source of RS in these cells.

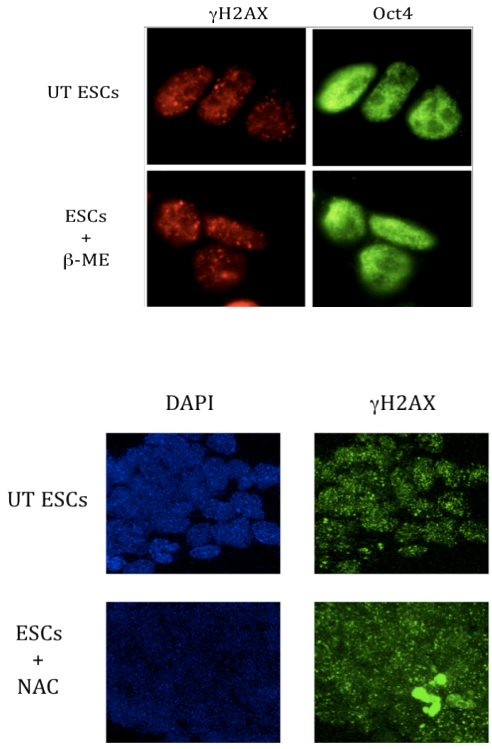


Figure 28: Oxidative DNA damage is not the source of ssDNA gaps in ESCs. Upper panel - single cell IF with Oct4 and γH2AX on untreated and 0.2mM β-ME treated ESCs after 24h. Lower panel - confocal microscopy images of untreated ESC colony and NAC (10mM, 10h) treated ESC colony stained with γH2AX. In both cases, there is no clear difference in γH2AX foci pattern/intensity between untreated and treated ESCs.

3.3.2 Hypoxia

Since ESCs reside within the inner cell mass of blastocysts, it could be hypothesized that they do not receive proper oxygenation due to lack of extensive vascularization during this developmental stage of the organism. To test whether hypoxia could represent the source of ssDNA accumulation (see 1.2.2.6) in ESCs, we cultivated cells in normoxic and hypoxic conditions for 2 passages. Again, there was no visible difference in the γH2AX staining pattern/intensity between the two conditions, ruling out the role of hypoxic conditions causing ssDNA gaps in ESCs (Fig 29).

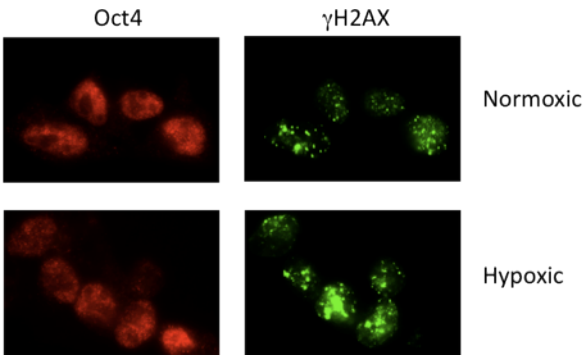


Figure 29: Hypoxia does not lead to ssDNA break formation in ESCs. Single cell IF of Oct4 and γH2AX in ESCs grown in normoxic (upper panel) and hypoxic conditions (lower panel) for 2 passages. ESCs stain positive for γH2AX in both conditions.

3.3.3 Collision between transcription and replication

Next, we tested whether increased interference of transcription with replication could possibly cause ssDNA gaps in ESCs as has been shown in other systems earlier (see 1.2.2.5). To this end, we transiently blocked transcription using the RNA chain elongation inhibitor cordycepin. We used 5-ethynyl uridine (EU) an analogue of uridine, as a marker of global transcription (#C10327, Life Technologies). As expected, the EU incorporation was abolished upon treatment with cordycepin (Fig 30, lower panel). However, instead of rescuing the stress phenotype, γH2AX levels increased marginally (Fig 31, upper panel) indicating that blocking transcription could pose additional problems that may be independent of replication in ESCs.

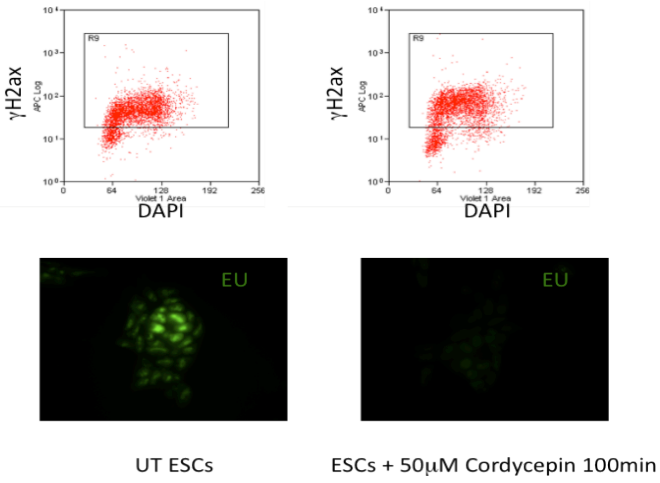


Figure 30: Interference between transcription and replication does not cause RS in ESCs. Upper panel - monitoring γH2AX levels by flow cytometry in untreated and cordycepin treated (50μM, 100min) ESCs. Treatment with cordycepin does not reduce ssDNA accumulation. Lower panel - EU incorporation visualized by IF in untreated and cordycepin treated ESCs. Cordycepin inhibits transcription (monitored by loss in EU incorporation).

3.3.4 Active DNA demethylation

A series of recent reports implicate the role of BER factors in active demethylation at distal elements in ESCs. Removal of methylated and/or oxidized bases by the BER machinery may involve transient generation of nicks/gaps on the DNA backbone, which looked as a likely source of the endogenous ssDNA gaps observed in ESCs (see 1.4.2).

5mC is first oxidized to 5hmC, which is further oxidized to 5fC and 5caC. This step is mediated by Tet1/2 family of dioxygenases. 5fC and 5caC are substrates of TDG, which removes the modified bases and brings about demethylation. We tested whether absence of Tet1/2 would prevent formation of 5fC and 5caC and hence block demethylation, therefore preventing formation of ssDNA gaps. However, Tet1/2 knockout (KO) ESCs exhibited γH2AX foci at comparable levels to WT ESCs (Fig 31).

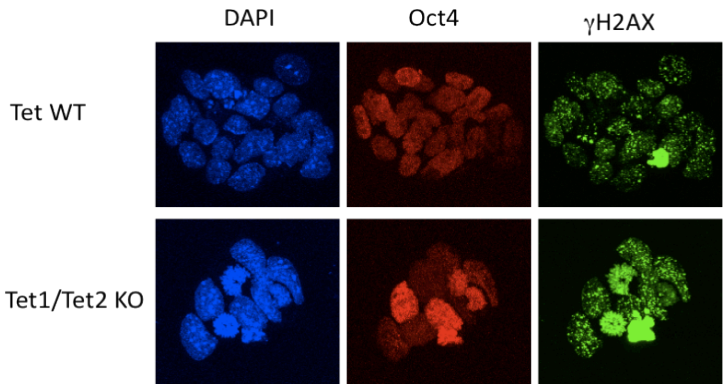


Figure 31: γH2AX foci do not depend on Tet1/Tet2 status in ESCs. Confocal images of WT and Tet1/2 KO ESCs stained with Oct4 and γH2AX. There is no clear difference in γH2AX foci pattern/intensity between WT and Tet1/2 KO cells. KO is short for knockout.

A recent study identified formation of 5hmC, 5fc and 5caC independently of Tet1/2, which would imply that Tet1/2 may not be the sole factors responsible for 5mC oxidation (Dawlaty et al., 2012). Hence, 5hmC and its further oxidized forms could still be produced in ESCs that serve as substrates for TDG. To test this possibility, we directly compared γH2AX foci pattern in mock transfected and TDG depleted ESCs. However, the staining pattern was again indistinguishable between the two samples (Fig 32, upper panel). Although the efficiency of TDG knockdown was quite high (Fig 32, lower panel), it is possible that undetectable levels of the protein are still able to exert its physiological function. Therefore, we compared γH2AX staining in WT and TDG KO ESCs. As shown in the upper panel in Fig 32, TDG KO

ESCs exhibit the same staining pattern as WT ESCs, thus reinforcing the conclusion that TDG-dependent demethylation is not a major source of gaps observed in unperturbed ESCs.

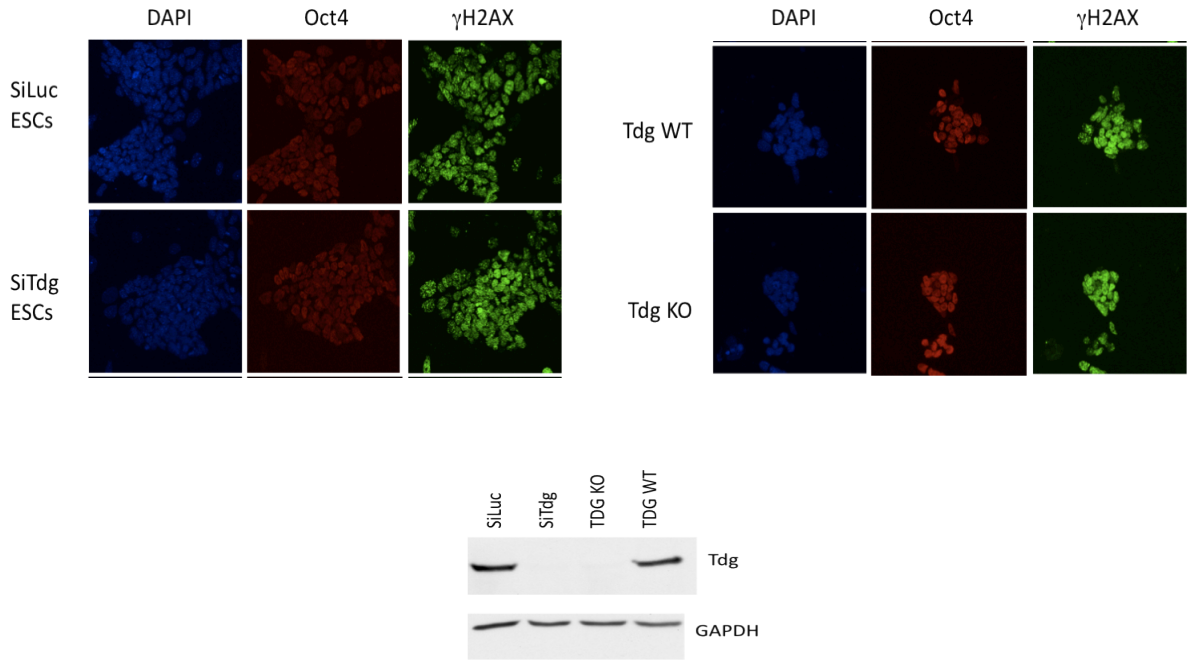


Figure 32: Active DNA demethylation mediated by TDG is not the source of gaps in ESCs. Upper panel - confocal images of mock transfected, TDG depleted, WT and TDG KO ESCs stained with Oct4 and γH2AX. There is no visible difference in γH2AX foci pattern/intensity between the different samples. Lower panel - detection of TDG protein levels in all four samples by Western Blotting. GAPDH is used as loading control.

Yet another possibility that encompasses excision of incorrect/misincorporated bases via the BER pathway at promoters in ESCs is that another putative glycosylase could mediate removal of modified bases. Irrespective of which glycosylase mediates the excision, the endonuclease Ape1 is then required to create nicks in the phophodiester backbone of DNA, which enables eviction of incorrect bases by the responsible glycosylase. Also, the redox function of Ape1 activates other enzymes involved in BER. Hence, should the ssDNA nicks result from BER-related events, inhibiting Ape1 activity should prevent their formation. We treated WT ESCs with the Ape1 inhibitor methoxyamine hydrochloride and observed no obvious reduction in γH2AX staining, substantiating the conclusion that BER does not mediate RS in ESCs (Fig 33).

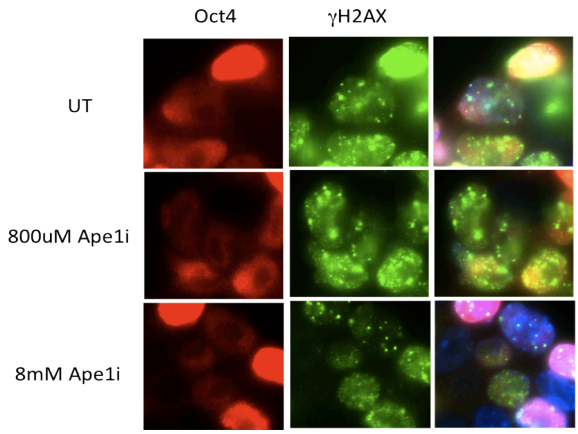


Figure 33: Ape1 mediated nicks during BER are not the source of γH2AX in ESCs. Oct4/γH2AX IF images of untreated ESCs and ESCs treated with indicated doses of Ape1 inhibitor for 12h. Treatment of ESCs with increasing concentrations of the inhibitor has little effect on ssDNA gaps.

One report has demonstrated that XPG mediates demethylation at the Oct4 promoter in *Xenopus laevis* (see 1.4.3). XPG has been traditionally described as an enzyme in the NER pathway that assists in removal of heavy adducts that are formed upon UV damage. In addition, XPG shows affinity for ssDNA and possesses endonuclease activity. However, as shown in Fig 34, effective XPG downregulation in ESCs had no detectable effect on γH2AX staining. Altogether, these results strongly suggest that ssDNA formation in ESCs is not related to DNA incision events associated with active DNA demethylation.

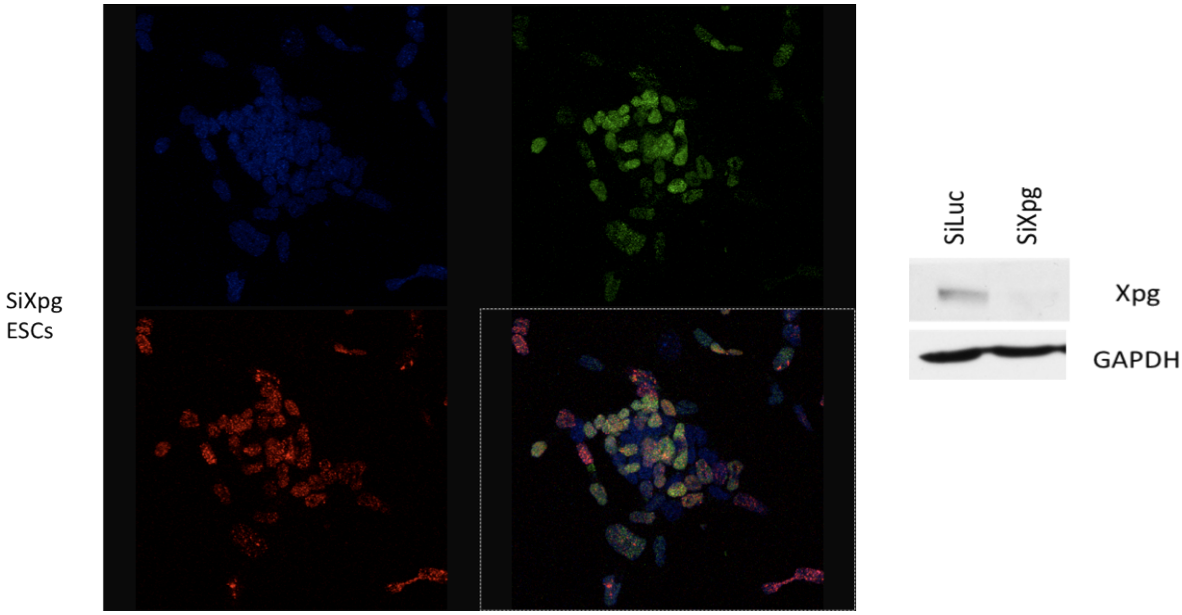


Figure 34: Active DNA demethylation mediated by XPG is not the source of gaps in ESCs. Left panel - confocal images of XPG depleted ESCs stained with Oct4 and γH2AX. ssDNA gaps persist even upon depletion of XPG. Right panel - detection of XPG protein levels in mock transfected and XPG depleted samples by Western Blotting. GAPDH is used as loading control.

3.3.5 Peculiarity in ESC cycle and altered origin licensing/firing

ESCs have short gap phases and spend most of their time in the S phase, which distinguishes them from other somatic cells. In addition, cell cycle regulators like Cdc25a, Cyclin E and c-Myc - which are also known oncogenes - are abundant in ESCs, (see 1.3.1.3). We tested whether RS in ESCs is due to the rapid G1-S transition, which is attributed to the constitutive over-expression of Cdc25a. Although knocking down Cdc25a does cause a marked reduction in EdU incorporation and disturbs cell cycle kinetics (Fig 35, lower panel), it does not suppress RS-associated γH2AX levels in ESCs (Fig 35, upper panel).

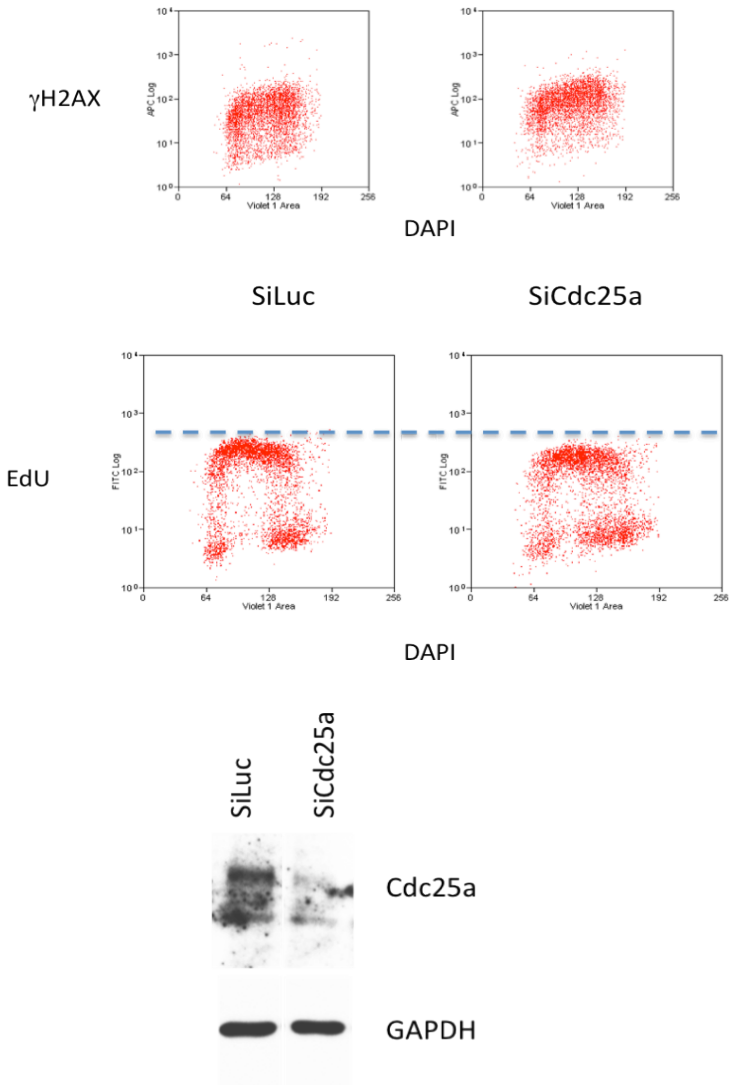


Figure 35: 'Uncontrolled' G1-S transition due to constitutively active Cdc25a does not lead to ssDNA break formation in ESCs. Upper panel - flow cytometry analyses of γH2AX and EdU levels in mock depleted and Cdc25a depleted samples. There is no difference in γH2AX levels between control transfected and Cdc25a depleted ESCs. However, there is a marked reduction in EdU incorporation upon Cdc25a knockdown. Lower panel - detection of Cdc25a protein levels by Western Blotting. GAPDH is used as loading control.

Recent reports (Bester et al., 2011, Jones et al., 2012) show that nucleotides are rate limiting during oncogene mediated RS (see 1.2.2.2). Due to their high proliferative capacity, it is plausible that ESCs exhaust the locally available nucleotide pool, indirectly leading to RS. However, supplementing culturing media with excess nucleotides also had no detectable effect on γH2AX levels in ESCs (Fig 36, upper panel). Since exogenous nucleotides compete with EdU during proliferation, reduction in EdU incorporation can be taken as a direct internal control for effective nucleotide incorporation (Fig 36, lower panel).

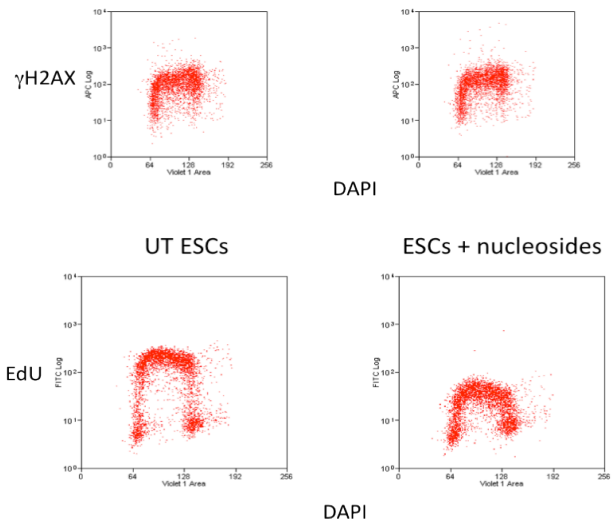


Figure 36: Exogenous addition of nucleotides does not rescue RS in ESCs. Upper panel - flow cytometry analyses of γH2AX levels in untreated and ESCs applied with excess nucleotides. There is no effect on ssDNA gaps upon exogenous addition of nucleotides. Lower panel - EdU incorporation in the respective samples. A marked reduction in EdU incorporation is observed since nucleotides compete with EdU.

Importantly, levels of the crucial origin licensing factor Cdc6 are much higher in ESCs compared to differentiating cells (Fig 37). Hence, we tested whether over-licensing maybe the source of RS in ESCs, similarly to what recently reported by our lab upon perturbations of the licensing program in somatic cells (Neelsen et al., 2013b). To this end, we tried depleting Mcm3, Cdt1, Cdc6 and the combination of the three and assessed the possible effects on γH2AX levels in ESCs. Mcm3 and Cdt1 proved difficult to knock down, although Cdc6 levels were successfully reduced upon siRNA mediated depletion (Fig 39). Although the capability of ESCs to differentiate is reduced upon knocking down origin licensing factors (compare cell densities highlighted in blue boxes), it has no effect on gaps as seen in Fig 38, upper panel. Despite efficient Cdc6 depletion in ESCs, there is no effect on the EdU positive

fraction (Fig 38, lower panel) - suggesting that low levels of the protein are probably sufficient for effective origin licensing.

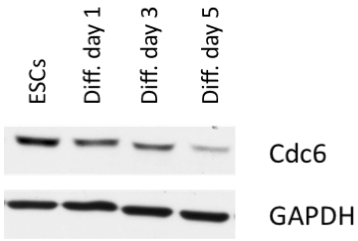


Figure 37: Evident decrease in Cdc6 protein levels upon differentiation of ESCs. Cdc6 levels in ESCs and after 1, 3 and 5 days of induction of differentiation. Diff. is short for differentiating. GAPDH is used as loading control.

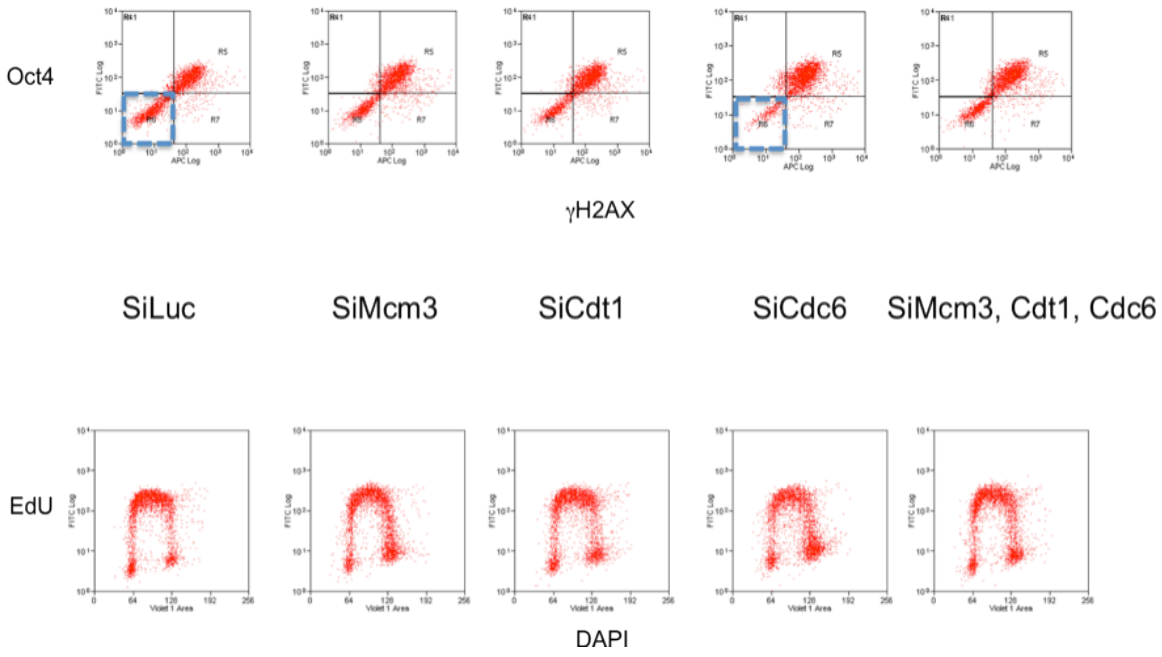


Figure 38: Knocking down licensing factors has no effect on ssDNA gaps or proliferation in ESCs. Upper panel - flow cytometry analyses of Oct4 vs. γH2AX positive/negative cells in mock depleted ESCs and ESCs depleted of the indicated licensing factors. There is no obvious difference between SiLuc transfected and SiMcm3/Cdt1/Cdc6 transfected samples in terms of γH2AX levels. Blue boxes: percentage of differentiating cells negative for both Oct4 and γH2AX in control transfected and Cdc6 depleted samples. Note the reduction in number of differentiating cells upon Cdc6 depletion. Lower panel - cell cycle analyses of aforementioned samples. Knocking down licensing factors has little effect on EdU incorporation in ESCs. This experiment has been reproduced at least thrice.

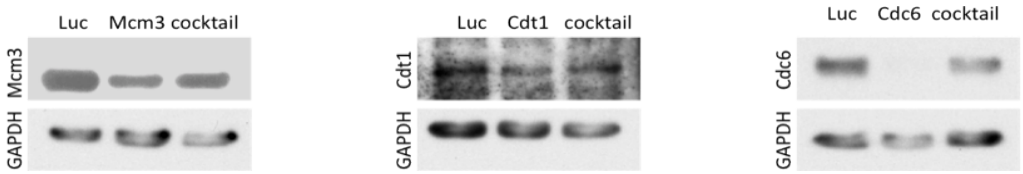


Figure 39: Detection of protein levels in mock transfected and respective licensing factor depleted samples. SiCocktail is a mixture of SiMcm3, SiCdt1 and SiCdc6. GAPDH is used as loading control.

Conversely, we considered the hypothesis that, despite the abundant levels, origin licensing factors may still not be sufficient for full replication in the fast ESC cell cycle. Hence, we tried pushing licensing further by indirectly over-expressing Cdt1 via Emi1 downregulation (see 1.2.2.3). Deregulation of licensing via Emi1 depletion (or Cdt1 overexpression) in other somatic cells leads to a marked reduction in EdU incorporation and brings about an increase in proportion of cells with more than 4N DNA content (Neelsen et al., 2013b). However, Emi1 depletion does not affect EdU incorporation in ESCs and causes re-replication only in a very small proportion of cells (Fig 40, upper panel). Further, knocking down Emi1 has little effect on ssDNA gaps in ESCs (Fig 40, lower panel). Differently from the somatic cells, Emi1 depletion in ESCs does not stabilize Cdt1 levels (Fig 41, right panel). Overall, these data suggest that origin licensing is robustly upregulated in ESCs and that affecting licensing levels in this system is particularly challenging and/or unlikely to result in major perturbations of the replication program. These results are further discussed in chapter 5. We are currently collaborating with J. Mendez's group (CNIO, Madrid) to directly compare DDR between WT and Cdt1- and/or Cdc6- overexpressing ESCs and blastocysts.

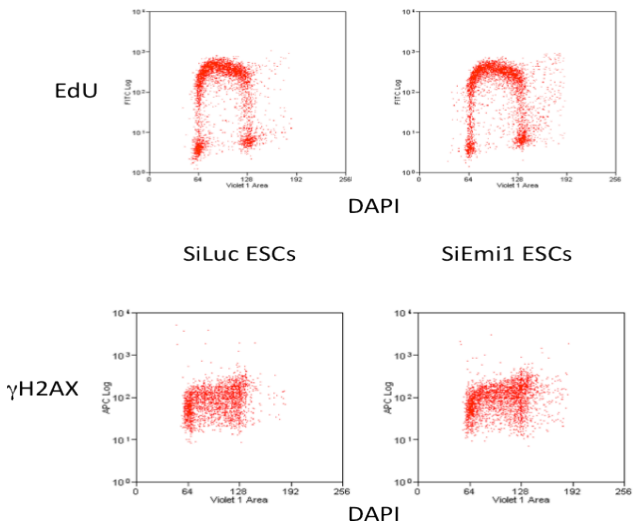


Figure 40: Deregulation of Emi1 has no effect on ssDNA gaps or proliferation in ESCs. Upper panel - EdU incorporation in mock transfected and Emi1 depleted ESCs monitored by flow cytometry. Lower panel - γH2AX levels in indicated samples. There is no apparent difference in EdU or γH2AX levels between control and Emi1 depleted ESCs.

Figure 41: Downregulation of Emi1 does not stabilize Cdt1 protein levels. Left panel - Emi1 protein levels in mock transfected and Emi1 depleted ESCs as detected by Western blotting. TFIIH is used as loading control. Right panel - Cdt1 levels in indicated samples. GAPDH is used as loading control. Cdt1 levels remain largely unaffected upon Emi1 depletion in ESCs.

While origin licensing appears upregulated in ESCs, evidence from at least two independent groups shows that origin firing is generally rate limiting in eukaryotes and more particularly during embryonic development (see 1.2.2.3). Thus, to test if origin firing factors are limiting in ESCs, we inhibited Cdc7 and knocked down Cdc45 (see 1.1.2 and 1.2.2.3). As shown in Fig 42, we observe a marked decrease in EdU incorporation coupled to a mild but significant increase in γH2AX in both scenarios. Interestingly, Yoshida et al. show that overexpressing the firing factors Sld3, Sld7 and Cdc45 in budding yeast rescues RS due to absence of rDNA licensing and Collart et al. demonstrate that origin firing is minimal during midblastula transition in *Xenopus*. Hence, we transiently over-expressed Cdc45 in ESCs. To our surprise, we observed a 1.7 fold reduction in γH2AX levels in ESCs that express 1.7 fold higher Cdc45 (Fig 43). Although these results are preliminary, there seems to be a linear relationship between Cdc45 levels and ssDNA break formation in ESCs. In conclusion, limited firing is a promising cause of RS in ESCs and its titration is tightly regulated spatiotemporally.

Based on these results we hypothesized that firing might be limiting in ESCs to effectively complete a round of DNA replication, especially considering the reduced time in the gap phases to fill residual unreplicated regions and ssDNA gaps, prior to the onset of a new replication round (see discussion). To directly test this hypothesis, we treated ESCs with the general CDK inhibitor roscovitine. As CDK activity is required for both G1/s and G2/M transitions, transient CDK inhibition markedly increases the relative length of ESC gap phases. Importantly, this perturbation also brings about a marked reduction in γH2AX levels throughout the ESC cycle, without affecting detection of the pluripotency marker Oct4 in the majority of the cells that lose γH2AX (Fig 44).

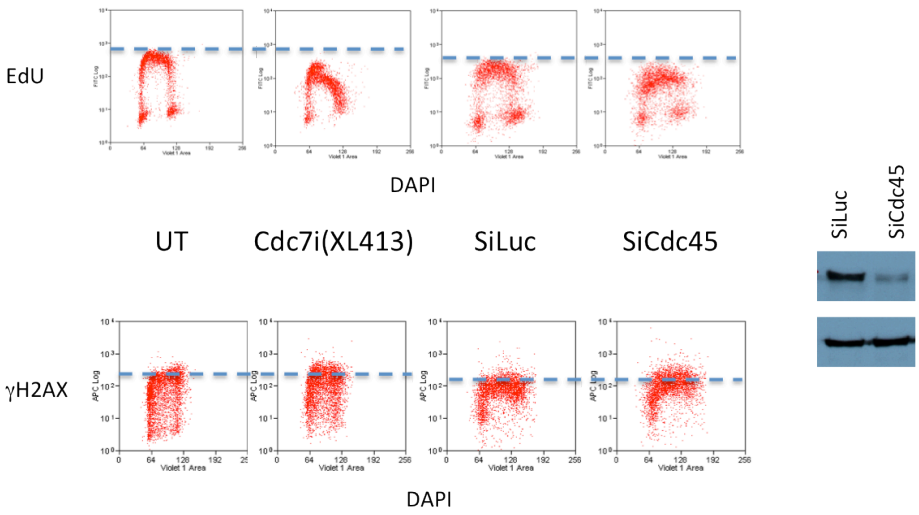


Figure 42: Inhibiting origin firing by either using small molecule inhibitor against Cdc7 or knocking down Cdc45 causes further increase in ssDNA gaps. Upper panel - EdU incorporation in untreated ESCs, ESCs treated with Cdc7 inhibitor, mock transfected and Cdc45 depleted ESCs monitored by flow cytometry. Lower panel - γH2AX levels in indicated samples. Cdc7 inhibition or Cdc45 depletion causes a marked reduction in EdU incorporation and a modest increase in γH2AX levels. Far right panel - Cdc45 levels in mock transfected and Cdc45 depleted ESCs detected by Western Blotting.

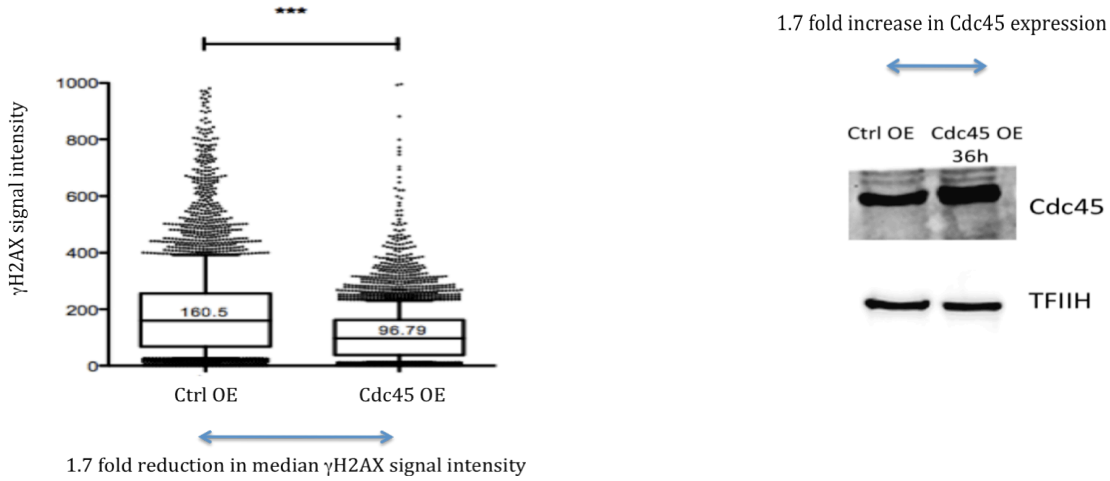


Figure 43: Cdc45 overexpression reveals a linear inverse relationship between Cdc45 levels and γH2AX signalling in ESCs. Left panel - Mann Whitney test for difference in γH2AX signal intensity between control and Cdc45 overexpressing samples collected 36h post transfection. Median of both populations indicated within the box plot. *** corresponds to P value < 0.0001. Overexpression of Cdc45 brings about a 1.7 fold reduction in endogenous ssDNA gaps in ESCs. Right panel - Cdc45 protein levels in control and Cdc45 overexpressing (1.7 fold) ESCs detected by Western Blotting 36h post transfection. Quantification of protein levels was performed using ImageJ.

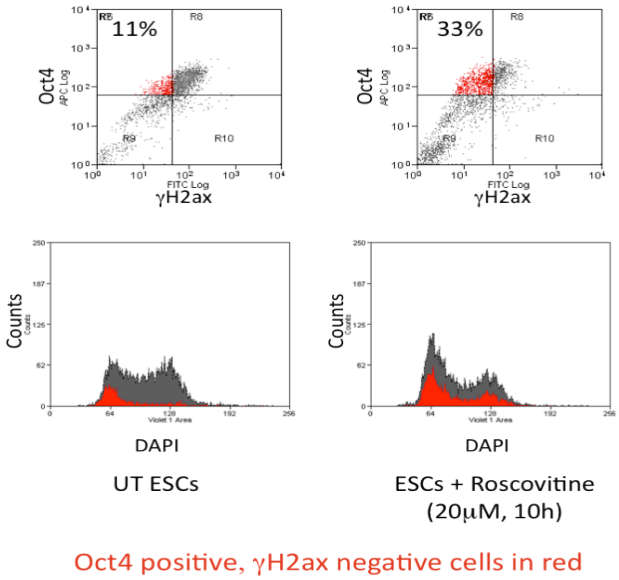


Figure 44: CDK inhibition lengthens ESC cycle and suppresses endogenous gaps. Upper panel - Oct4 vs. γH2AX levels in untreated ESCs and ESCs treated with 20μM roscovitine for 10h. A large proportion (33%) of Oct4 positive cells lose γH2AX after treatment with roscovitine. Lower panel - cell cycle analysis of untreated and roscovitine treated ESCs. Treatment with roscovitine increases gap phase length in ESCs.

Altogether, these results strongly suggest that a combination of limiting origin firing and reduced length of gap phases underlies the accumulation of unreplicated regions in cycling ESCs, leading to a number of parallel RS phenotypes, typically associated with replication of a discontinuous template.

4. OTHER PRELIMINARY RESULTS

4.1 DDR in iPSCs

We also attempted to investigate DDR during iPSC generation, which encompasses overexpression of the four factors Oct4, Klf4, Sox2 and c-Myc. These factors comprise also known oncogenes and the process of reprogramming has been associated with accumulation of genetic instability (see 1.3.2 and 1.3.4). Due to the low reprogramming efficiency, it was technically difficult to identify the small and scattered subpopulation of MEFs that would eventually gave rise to iPSC colonies. However, when we stained the reprogrammed iPSC colonies, the Oct4 positive cells consistently exhibit γH2AX foci similarly to ESCs (Fig 45). Interestingly, a recent study identifies reorganization of replication timing during iPSC reprogramming irrespective of the procedure employed (Lu et al., 2014). However, whether

γH2AX foci in iPSCs result from specific alteration of the replication initiation program or are simply due to the reprogramming procedure remains to be determined.

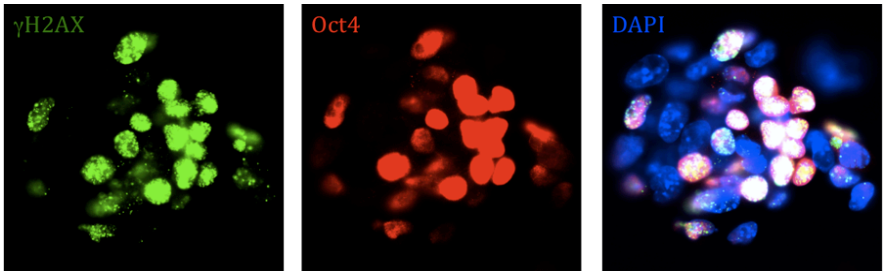


Figure 45: iPSCs exhibit γH2AX foci similar to ESCs. IF analysis with indicated makers - iPSC colony arising 20 days post reprogramming. The cells that acquire Oct4 are also co-positive for the DDR marker γH2AX.

4.2 DDR in quiescent vs. active HSCs

As the term suggests, quiescent HSCs are mainly dormant and are activated upon injury, stress or inflammation (see 1.3.3). Based on our observations on highly proliferating ESCs, we postulated that DDR signaling could be quite distinct in non-cycling vs. cycling HSCs and established methods to investigate it.

4.2.1 Increase in HSC proliferation upon pI:C treatment

Since the main aim of our study was to study replication features and associated DDR in HSCs, we compared quiescent HSCs with activated ones. Mice were injected with PBS (control group) and polyinosinic-polycytidylic acid (pI:C) and their total bone marrow (TBM) was isolated. The TBM was then used to sort out HSCs (Lin^- Sca^+ $C\text{-}Kit^+$ $CD34^-$ $Flk2^-$). pI:C activates IFN-a, which in turn activates dormant HSCs (Essers et al., 2009). As shown in Fig 46, pI:C specifically pushes HSCs into proliferation and has no effect on the TBM.

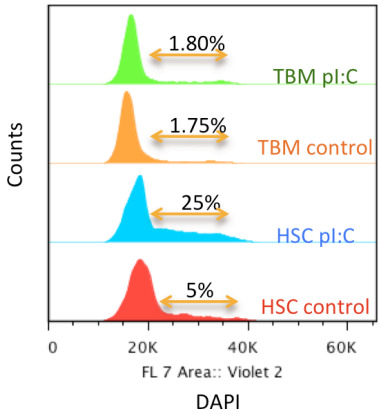


Figure 46: pI:C treatment specifically activates HSCs. Flow cytometry analysis of percentage of S phase cells in untreated and pI:C treated mice. The number of TBM cells in the S phase from mice treated with polyinosinic-polycytidylic acid (pI:C) is similar to that in the control group. However, the percentage of cycling HSCs from pI:C treated mice is 5x higher than the control group.

4.2.2 Detection of γH2AX in activated HSCs

Untreated HSCs display reduced EdU incorporation compared to TBM and cycling HSCs (EdU positive) only occasionally exhibit γH2AX (Fig 47, third plot from left). Noticeably, post pI:C treatment, almost all activated HSCs accumulate γH2AX (Fig 47, last plot). Although these should be considered as preliminary results (the experiment has been performed only once), we can hypothesize that HSCs experience RS when they exit dormancy and are faced by the immediate need to proliferate. Furthermore, similar results have been recently obtained in the Manz lab when mice were challenged with LPS - i.e., HSCs from mice treated with LPS accumulate punctuate γH2AX foci (Takizawa et al., manuscript in preparation). Hence, irrespective of how HSCs are stimulated, they seem to display DDR activation.

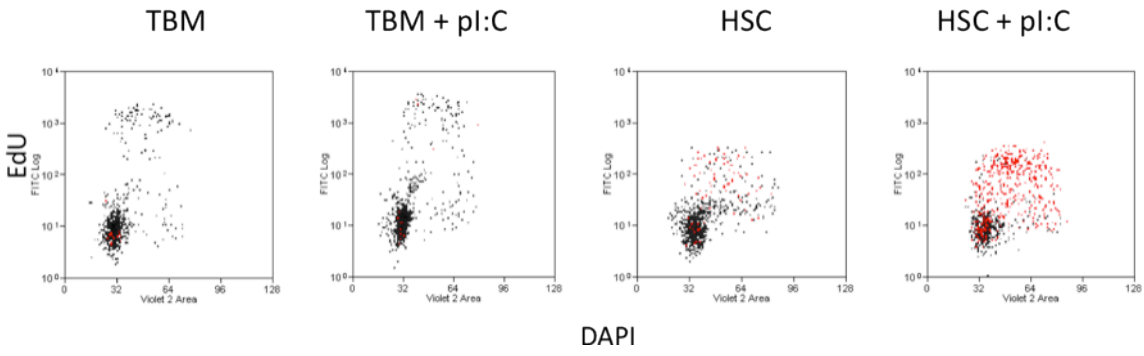


Figure 47: Untreated HSCs exhibit reduced proliferative capacity and display active DDR upon pI:C stimulation. Cell cycle analysis of TBM and HSCs by flow cytometry. γH2AX positive cells in red. HSCs exhibit reduced EdU incorporation in comparison to TBM. A few control HSCs are positive for γH2AX in the absence of any stimulation. Upon pI:C stimulation, most HSCs stain positively for γH2AX.

5. DISCUSSION

DNA replication is an essential cellular process, which involves duplication of the genetic material. As mentioned already in this thesis, various impediments may arise during the course of replication that might hinder replication fork progression, leading to RS. The DDR pathway helps in genome surveillance and in the removal/bypass of such obstacles to allow for replication to continue. This pathway is particularly important in stem cells, which are capable of eventually giving rise to all other cells that form the organism.

DDR in ESCs

ESCs possess the ability to differentiate into the three distinct germ layers, and are therefore pluripotent. The control of pluripotency has to be tightly regulated and the self-renewal capacity has to be maintained before ESCs are programmed to differentiate. The dynamics of the development process have to be coordinated in a precise manner since ESCs need to rapidly proliferate and differentiate in a limited time span. Several reports implicate specific cell cycle adaptations in ESCs to be, at least in part, responsible for their pluripotent status. Intriguingly, Geminin - one the factors that is required for preventing over-replication in proliferating cells by inhibiting the initiation factor Cdt1 - is also required for the establishment of pluripotent cells (Gonzalez et al., 2006). Unlike in other somatic cells, Geminin is present during the G1 phase of the ESC cycle and regulates pluripotency (Yang et al., 2011b). In contrast, Cdc6, another initiation factor that mediates replication origin licensing with Cdt1, does not oscillate and is constitutively expressed throughout the ESC cycle. Furthermore, Cdc6, CDKs and Cyclins are all extremely abundant in ESCs compared to MEFs (Fujii-Yamamoto et al., 2005). Hence, it is fully plausible that replication is differentially regulated in ESCs and evolutionary adapted to occur within the restrictive conditions imposed by rapid ESC proliferation.

In line with previous reports, we show that ESCs exhibit punctuate γH2AX foci that do not co-localize with the specific DSB marker 53BP1 and are therefore not indicative of DNA DSBs. However, γH2AX positive ESCs are also positive for RPA and Rad51, both ssDNA binding proteins that are recruited to chromatin and visualized in IF stainings only upon exposure of extensive ssDNA regions. These data strongly implicate that ESCs accumulate

discontinuities in their rapidly replicating DNA. Blastocysts derived from mice also show robust γH2AX and RPA staining patterns, emphasizing that the discontinuities detected in cultivated ESCs are a true reflection of the *in vivo* situation. Indeed, direct visualization by electron microscopy confirms that the vast majority of the replication intermediates in ESCs do display ssDNA gaps/nicks. In agreement with previous studies from our lab, ssDNA accumulation on the template is accompanied by increased replication fork reversal in ESCs. Furthermore, replication fork progression in ESCs is markedly slower compared to somatic cells. Hence, albeit principally counterintuitive for cells priming the development of an entire organism, ESCs do experience endogenous RS - their replication intermediates exhibit a number of molecular features reminiscent of those induced exogenously upon treatment with genotoxic drugs. One of the most striking observations in this thesis is that all these hallmarks of RS rapidly disappear upon induction of differentiation, as soon as the stem cell marker (Oct4) is lost, well before cells stop proliferating. Thus RS is an inherent characteristic of stem cells, and not solely a consequence of rapid proliferation.

Apart from its role in HR and DSB repair, Rad51 is also known to modulate replication fork progression (Hashimoto et al., 2010) and to protect stalled forks from being degraded (Schlacher et al., 2012). Therefore, the increased amounts of Rad51 reported in ESCs could serve to remodel replication forks in face of RS and to protect them from breakage, rather than to repair forks once they have collapsed. In line with this, yet unpublished results from the lab strongly suggest that Rad51 is strictly required for replication fork reversal in response to different genotoxic treatments (Zellweger et al., manuscript in preparation). Since the frequency of fork reversal drastically drops down upon differentiation, and so do Rad51 levels, it would be worth testing the role of Rad51 in fork reversal specifically in ESCs.

It was previously shown that γH2AX in ESCs is not dependant on the ATM or DNA-PKcs status (Banath et al., 2009). However, RS is mainly channelled through the ATR pathway. In agreement with the data described above, we now report that transient inhibition of ATR activity leads to a marked reduction in γH2AX staining, suggesting that ATR phosphorylates H2AX in ESCs; further substantiating that ESCs are constantly exposed to RS.

Drug induced RS has been thoroughly studied in the Lopes lab (Chaudhuri et al., 2012, Berti et al., 2013) and is being complemented by ongoing work (Chaudhuri et al., submitted;

manuscript attached to this thesis). The published studies focused on RS induced by Top1 poisons, like camptothecin (CPT), that prevent Top1 religation activity, thereby leading to ssDNA nick formation. Upon CPT-induced discontinuities, PARP activity becomes crucial in protecting forks and avoiding chromosomal breakage, via stabilization of replication forks in the reversed state (Chaudhuri et al., 2012, Berti et al., 2013). Recent evidence has extended the same concept to a whole series of different genotoxic drugs, frequently used in cancer chemotherapy (Zellweger et al., manuscript in preparation). Overall, fork reversal is emerging as an evolutionarily conserved mechanism to prevent DSB formation, coupled to protective replication fork slowdown in face of lesions or discontinuities on the DNA template (Neelsen and Lopes, *in press*). In keeping with this model, this study reveals that replication fork reversal is particularly frequent in unperturbed ESCs and that PARP1 activity is required to prevent replication fork collapse and chromosomal breakage in ESCs even in the absence of any exogenous damage - most likely because of ssDNA gaps/nicks inherently present in these cells.

As many of the observations discussed above descend from the accumulation of ssDNA nicks and gaps on ESC chromosomal DNA, one of the crucial biological questions I had to address was the source of these DNA lesions. To investigate why ESCs accumulate template discontinuities and experience RS, a number of different hypotheses were tested and excluded during the course of this thesis. In brief, RS in ESCs is not due to culture conditions, hypoxia, oxidative DNA damage, collision between transcription and replication or limiting nucleotide levels. Once these simpler hypotheses had been excluded, active DNA demethylation - which is reportedly frequent in ESCs during epigenetic reprogramming and involves the removal of modified bases via creation of nicks/gaps - seemed like the most plausible source of discontinuities on ESC DNA molecules. Hence, a considerable amount of time was spent pursuing this model and testing it via a number of different tools. Briefly, ssDNA gaps persist in TDG depleted or TDG knockout ESCs, Tet1/Tet2 double knockout ESCs, and remain indifferent to Ape1 inhibition or XPG depletion in ESCs. Thus, taken together, this study also demonstrates that active demethylation mediated either by the BER or the NER pathway is not the underlying source of RS and DDR in ESCs.

Apart from being abundant, replication licensing factors are quite resilient in ESCs. Knocking them down by siRNA proved to be quite difficult and partial depletion had little effect on replication/proliferation. A recent high-impact report shows that licensing factors are also

markedly upregulated during early embryonic development in *Xenopus laevis*. However, despite higher levels of licensing factors, the origin firing factors Treslin, Cut5, RecQL4 and Drf1 are rate limiting during the mid-blastula transition (Collart et al., 2013). As shown in this study, deregulating origin licensing either by partially depleting Cdc6/Cdt1/Mcm2 levels or by knocking down the negative regulator of licensing, Emi1, had no detectable effect on the endogenous DDR signalling (γH2AX) in ESCs. However, inhibiting origin firing either by using a specific Cdc7 inhibitor or by knocking down Cdc45 leads to a modest but significant increase in DDR signalling, suggesting that origin firing might indeed be rate limiting also in ESCs. Most notably, preliminary experiments show that mild overexpression of a single origin firing factor, i.e. Cdc45, is sufficient to partially rescue endogenous γH2AX in ESCs. Whether other firing factors (e.g., Treslin) are also rate limiting in ESCs and whether co-expression of these factors additionally suppresses DDR signalling and other molecular manifestations of RS in ESCs (i.e. fork slowing, ssDNA accumulation and fork reversal) will be addressed by additional experimental work within the next few weeks, which we consider essential for publication of this work.

It is worth mentioning that a previous report also identified ssDNA gaps in ESCs using an alternative assay (alkaline comet gels) and suggested incomplete replication in ESCs as the source of these discontinuities (Chuykin et al., 2008). However, this study did not elaborate on how these nicks/gaps could arise. It is now known that incomplete replication during S phase is not necessarily deleterious and is often dealt with during the subsequent gap phases/mitosis to maintain genome stability (Mankouri et al., 2013). As suggested in this study, despite abundant licensing, origin firing may be limiting in ESCs to cope with consecutive replication cycles temporally separated by only short gap phases. It is indeed plausible that under-replicated chromosomal regions, physiologically present in every S phase upon completion of bulk DNA replication, are not fully dealt with during the following phases of the cell cycle, owing to the time constraints and the cell cycle adaptations associated with the high proliferation rate of ESCs. In this scenario, ssDNA gaps and γH2AX signalling would be inherited from one replication round to the next and would thus persist throughout the ESC cycle (Fig 48).

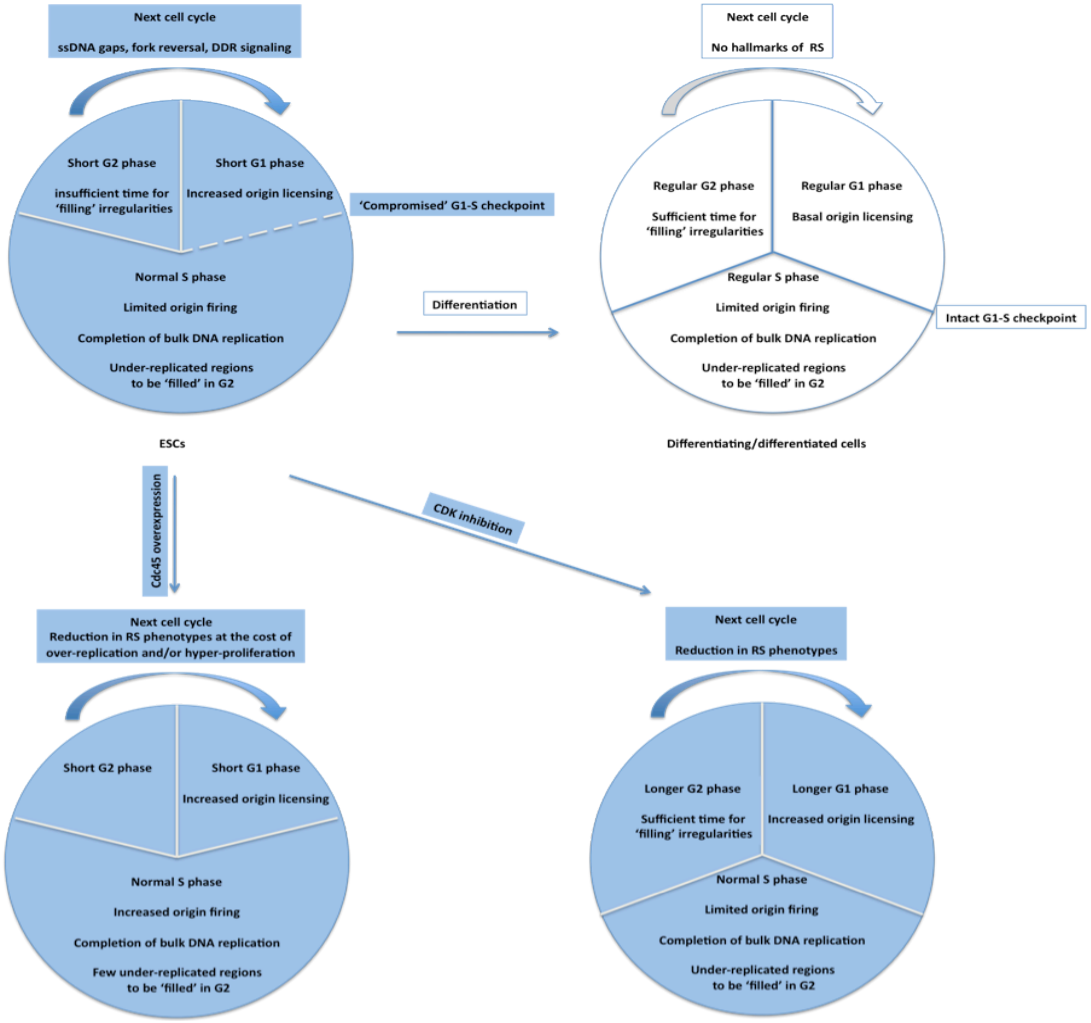


Figure 48: Graphical representation for explanation of endogenous replication stress in embryonic stem cells. Each pie chart represents an arbitrary unit of time. The lines within the pie chart mark boundaries of the respective cell cycle phases. The area enclosed between two lines is proportional to the time spent in that phase of the cell cycle. Abundant licensing during the short G1 phase in ESCs is followed, as in somatic cells, by firing of a limited number of origins in S phase, leading to late-replicating chromosomal regions that require extra-time for replication termination after bulk genome replication is complete. Differently from somatic cells, the extra time for replication completion before a new replication round is inherently limited in ESCs by particularly short gap phases, impairing resolution of under-replicated regions which persist and are therefore inherited by the next replication round. In these conditions, cycling of ESCs is consistently associated with ssDNA accumulation and other hallmarks of RS. Indeed, inhibiting CDK activity, and thereby increasing cell cycle length, suppresses these hallmarks. Extra-numerary firing events would in principle be able to compensate this defect by accelerating the replication process, but are likely to be counter-selected in evolution, because of the associated risks of over-replication, frequently found in cancer cells upon deregulation of the replication initiation apparatus. In differentiating/committed cells regular origin licensing and firing would be sufficient to assist complete replication, as under-replicated regions arising physiologically during S phase are dealt with during the subsequent gap phases of the cell cycle. Therefore, differentiating/committed cells do not display hallmarks of replication stress in the absence of exogenous genotoxic agents.

Studies from David Gilbert's group show that replication domains are dense and organized in compact clusters in ESCs, whereas their density is reduced and they are consolidated into larger clusters in differentiated cells (Hiratani et al., 2008). Hence, origin licensing seems to be upregulated in ESCs. In our study, we show that ESCs clearly accumulate ssDNA regions that possibly arise due to limited firing and thus, incomplete replication. However, a crucial biological question descending from this model is - why is origin firing not upregulated in accordance with the increased licensing in ESCs, so as to avoid inheritance of unreplicated DNA in consecutive replication cycles? As previously mentioned, upregulation of licensing factors, coupled to limiting firing factors, has been observed during early development in *Xenopus* (Collart et al., 2013) and thus possibly reflects evolutionary constraints to the replication process. Therefore, one possible explanation is that limiting levels of firing factors, in ESCs as in any other cell, may indeed be necessary to prevent over-replication, which is frequently observed in cancer cells and consistently associated with chromosomal breaks and rearrangements (Hook et al., 2007). Furthermore, the inner cell mass within the blastocyst (from where ESCs are derived) may limit origin firing also to prevent hyper-proliferation, owing to space constraints and the need to differentiate into other cell types within a limited time span. Thus, several evolutionary constraints, partly inherent to genome stability and partly to the developmental program, may have contributed to limit origin firing spatiotemporally in ESCs.

Effective firing with the given levels of initiation factors may be further limited in ESCs by the particularly short G1 phase. The conversion of the preRC to IC - a critical step that determines origin firing in the S phase - is indeed dependant on CDK and Cdc7 activity (see 1.1.2) and typically happens towards the end of the G1 phase. Indeed, we show that transient inhibition of CDK activity in ESCs, which prolongs cell cycle length and leads to ESC accumulation in G1 and G2 phases, markedly reduces H2AX phosphorylation in the following S phase.

An important implication of this study is that ESCs can retain their pluripotency even with reduced CDK and/or altered origin activity, as interfering with CDK activity/origin firing clearly affects γH2AX levels in ESCs without affecting Oct4 levels. Hence, future experiments can be designed to identify novel factors involved in cell cycle regulation and replication origin firing by simply monitoring reduction in γH2AX in pluripotent ESCs.

DDR in other stem cells

During reprogramming of somatic cells into stem cells, the genome is reorganized and replication timing is markedly altered (Lu et al., 2014). This finding is very much in line with the global reorganization of replication domains observed upon ESC differentiation (Hiratani et al., 2008). Besides the known resemblance with ESCs for numerous other parameters, our pilot experiments show that iPSCs also exhibit punctuate γH2AX foci. Hence, it is conceivable that iPSCs also experience RS, by mechanisms analogous to ESCs. As opposed to ESCs and iPSCs, HSCs are instead largely quiescent. However, preliminary experiments suggest that upon stimulation, activated HSCs also accumulate γH2AX during chromosomal replication. This potentially exciting observation will be pursued by extensive further experiments in the future, in order to assess whether the HSC cell cycle experiences similar limits and regulations as those addressed in ESCs in this study.

6. MATERIALS AND METHODS

6.1 Cell culture, media and supplements

ESCs were cultivated on feeder cells (MEFs inactivated with 10μg/ml mitomycin C (Sigma)) for at least two passages before performing experiments. ESCs were then separated from feeder cells by trypsinization and centrifugation and grown on gelatinized tissue culture dishes to avoid unwanted signals from contaminating feeder cells.

Feeder/differentiation medium:	Dulbecco's Modified Eagle Medium (Sigma) 15% Fetal Bovine Serum (Gibco) 1mM Sodium Pyruvate (Sigma) 1x Non-Essential Amino Acids (Sigma) 1x Penicillin-Streptomycin-L-Glutamine (Life Technologies) 0.1mM β-Mercaptoethanol (Sigma)
ESC medium:	Feeder medium + 1000U/ml LIF (Millipore)
N2B27 medium:	1:1 Neurobasal Medium:DMEM/F-12 (Life Technologies) 1xPenicillin-Streptomycin-L-Glutamine (Life Technologies) 1x N2-Supplement (Life Technologies) 1x B27 Supplement (Life Technologies) 0.05mM 2-Mercaptoethanol (Life Technologies)
N2B27 supplements (2i):	1μM PD0325901 (Stemgent) 3μM CHIR99021 (Stemgent)
Others:	0.1% Gelatin from porcine skin (Sigma) Trypsin EDTA (10x stock) (Sigma) PBS (w/o Ca and Mg) (in house, IMCR)

6.2 Cell lines

The following cell lines were used for this study:
E14 ESCs (provided by P. Cinelli, Clinic for Trauma Surgery, Zurich)
Stat3 ESCs (provided by P. Cinelli, Clinic for Trauma Surgery, Zurich)
JM8 ESCs (provided by P. Cinelli, Clinic for Trauma Surgery, Zurich)
Primary MEFs (provided by P. Cinelli, Clinic for Trauma Surgery, Zurich)
Immortalized MEFs (provided by F. Althaus, Institute of Pharmacology and Toxicology, Zurich)
WT and Tet DKO ESCs (provided by H. Stunnenberg, RIMLS, Nijmegen)
WT and TDG KO ESCs (provided by P. Schär, Department of Biomedicine, Basel)

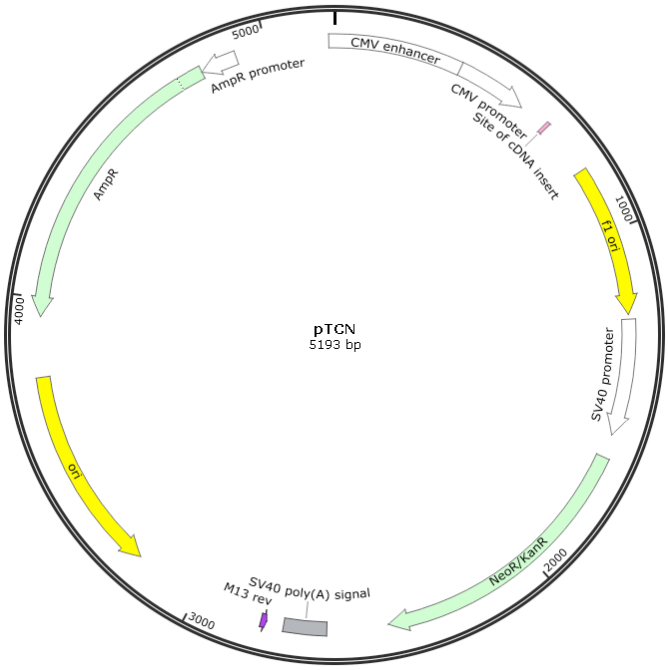
6.3 Immunofluorescene/confocal microscopy

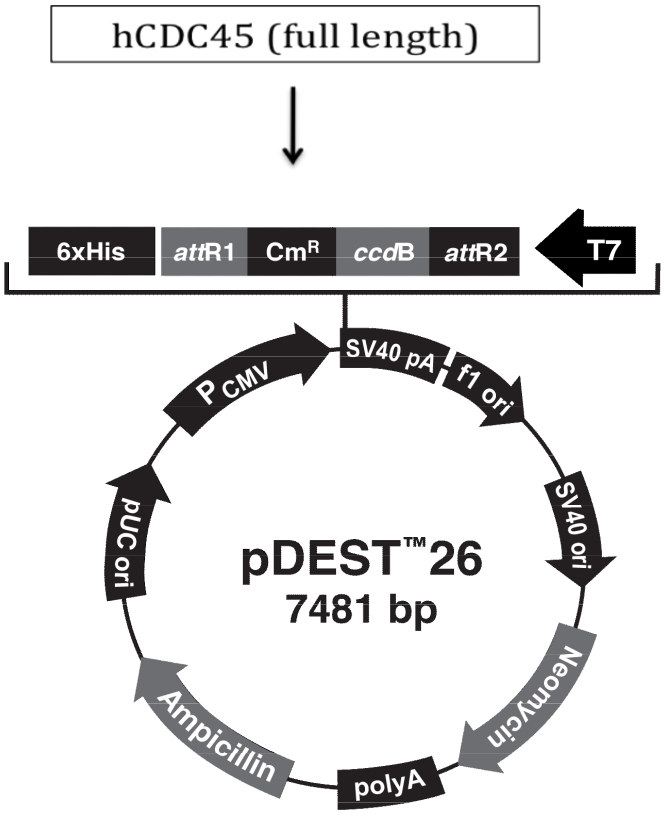
For immunofluorescence, cells were fixed with 4% formaldehyde/PBS, permeabilized with 0.5% TritonX-100, stained for γ-H2AX, Oct4, RPA, Rad51 and 53BP1 as indicated, detected by appropriate secondary antibodies, and mounted with Vectashield (Vector Laboratories). 0.1% PBST (0.1% Tween in 1xPBS) was used for washes after primary and secondary antibody incubations. Images were acquired at 63x, using Leica Application Suite 3.3.0. on a microscope (model DMRB; Leica) equipped with a camera (model DFC360 FX; Leica) or with a confocal microscope, Leica TCS SP5. Images were taken at 63x magnification using LAS AF.

6.4 Transfections and treatments

For knockdown experiments, cells were transfected 24-72h with the indicated siRNA using RNAiMax transfection reagent (Life Technologies) according to the manufacturer's instructions: siLUC (100-200 nM; 5′-GGUACGCGGAAUACUUCGAdTdT-3′), siTdg, siErcc5, siCdc6, siCdt1, siMcm3, siCdc45, siCdc25a, siDub3, siFBXO5 (100-200nM Smart pool Dharmacon).

For transient overexpression, the following plasmids were transfected for 36h using jetPrime (Polyplus transfection) according to manufacturers instructions: pTCN empty vector (provided by A. Müller, IMCR, Zurich), pDEST26-Cdc45 (provided by J. Mendez, CNIO, Madrid). Plasmid maps enclosed below:





The following inhibitors were used for the indicated time periods at mentioned final concentrations before collection:

Inhibitor (source)	**Duration of treatment**	**Final concentration**
ATR inhibitor ETP-46464 (provided by O. Fernandez-Capetillo, CNIO, Madrid)	12h	5μM
PARP1 inhibitor Olaparib (provided by S. Ferrari, IMCR, Zurich)	1h	10μM
Top1 inhibitor Camptothecin (Sigma)	1h	25nM
Reducing/scavenging agent N-acetylcysteine (Sigma)	10h	10mM
Transcription inhibitor Cordycepin (provided by P. Jansak, IMCR, Zurich)	100min	50μM
Ape1 inhibitor Methoxyamine hydrochloride (Sigma)	12h	800μM-8mM
Cdc7 inhibitor XL413 (provided by C. Santocanale, NCBES, Galway)	6h	10μM.
CDK inhibitor Roscovitine (Sigma)	10h	20μM.

6.5 Flow cytometry

For flow cytometric analysis for γ-H2AX/EdU/DAPI, cells were labeled for 30 min with 10μM EdU, harvested, and fixed for 15 min with 4% formaldehyde/PBS. Cells were washed with 1% BSA/PBS, pH 7.4, permeabilized with 0.5% saponin/1% BSA/PBS, and stained with anti–γ-H2AX antibody (#05-636; EMD Millipore) for 2 h, followed by incubation with a suitable secondary antibody for 30 min. Incorporated EdU was labeled according to the manufacturer's instructions (#C35002; Invitrogen). For flow cytometric analysis for γ-H2AX/Oct4/DAPI, cells were fixed and permeabilized as described above, followed by incubation with antibodies against γ-H2AX (#9718; Cell Signaling Technology) and Oct4 (BD Biosciences) and suitable secondary antibodies. In both assays, DNA was stained with 1μg/ml DAPI. Samples were measured on a Cyan ADP flow cytometer (Beckman Coulter) and analyzed with Summit software v4.3 (Beckman Coulter). For statistical analyses, Mann-Whitney test was applied to compute if differences in signal intensities were significant using Prism (GraphPad Software).

6.6 Western blotting

Cells were collected and either snap frozen in liquid nitrogen or immediately lysed using 2x Laemmli buffer. Protein amounts were normalized using known concentrations of BSA and protein absorbance was measured using Nanodrop technology. SDS-gels were run at 15-18 mA and proteins were either wet-blotted overnight (30V, 4°C) or for 2h (100V, 4°C) at room temperature on Hybond ECL transfer membrane (GE Healthcare). Membranes were blocked in 2% ECL (GE Healthcare) in 0.1% TBST (1xTBS supplemented with 0.1% Tween-20) for at least 30min and incubated with primary antibodies over night at 4°C or at room temperature for 4h in blocking solution and secondary antibodies were added for 1h at room temperature (in blocking solution). Membranes were washed 3 times with 0.1% TBST, 10' each, after primary and secondary antibody incubations and detected with ECL detection reagent (GE healthcare). Differences in protein levels were normalized against the loading control and the signal intensity was quantified using ImageJ.

6.7 Antibodies

The following primary antibodies were used: γ-H2AX (#05-636; EMD Millipore), 53BP1 (ab21083; Abcam), Oct3/4 ((#611203; BD Transduction Laboratories), RPA (NA19L; Calbiochem), Rad51 (sc-8349; Santa Cruz Biotechnology, Inc.), CycE (sc-198; Santa Cruz Biotechnology, Inc.), Cdc25A (sc-7389; Santa Cruz Biotechnology, Inc.), CHK1 pS345 (#2348; Cell Signaling Technology), CHK1 (sc-8408; Santa Cruz Biotechnology, Inc.), KAP1-pS824 (A300-767A; Bethyl Laboratories, Inc.), KAP1 (A300-274A; Bethyl Laboratories, Inc.), TFIIH (sc-293; Santa Cruz Biotechnology, Inc.), GAPDH (provided by A. Sartori, IMCR, Zurich), Cdc6, Cdt1, Mcm3, Mcm4, Cdc45 (provided by J. Mendez, CNIO, Madrid), Total and phosphoMcM2 (provided by C. Santocanale, NCBES, Galway). The secondary antibodies used were Alexa Fluor conjugates (Alexa Fluor 488, 594, and 647; Invitrogen) for flow cytometry and immunofluorescence and anti-rabbit and anti-mouse ECL (GE Healthcare) for Western blotting.

6.8 Pulse-field gel electrophoresis

Pulse-field gel electrophoresis was performed as follows: cells were embedded in 0.8% agarose plug (2.5×10^5 cells/plug), digested in lysis buffer (100 mM EDTA, 1% [wt/vol] sodium lauryl sarcosyne, 0.2% [wt/vol] sodium deoxycholate, and 1 mg/ml proteinase K) at 37°C for 48 h, and washed in 10 mM Tris-HCl, pH 8.0, and 100 mM EDTA. Electrophoresis was performed at 14°C in 0.9% (wt/vol) Pulsed Field Certified Agarose (Bio-Rad Laboratories) containing Tris-borate/EDTA buffer in a CHEF DR III apparatus (9 h, 120°, 5.5 V/cm, 30–18 s switch time; 6 h, 117°, 4.5 V/cm, 18–9 s switch time; 6 h, 112°, 4 V/cm, 9–5 s switch time; Bio-Rad Laboratories). The gel was stained with ethidium bromide and imaged on an Alpha Innotech Imager.

6.9 DNA fiber spreadings

Cells were sequentially pulse-labeled with 30 μM CldU and 250 μM IdU for 20 min each and harvested. Cells were then lysed and DNA fibers stretched onto glass slides by tilting them. The fibers were then denatured with 2.5 M HCl for 1 h, washed with PBS, and

blocked with 0.2% Tween 20 in 1% BSA/PBS. CldU and IdU tracks were detected with anti-BrdU antibodies recognizing CldU (ab6326; Abcam) and IdU (347580; BD), respectively, and appropriate secondary antibodies. Images were acquired with a microscope (model DMRB; Leica) equipped with a camera (model DFC360 FX; Leica). Images were taken at 63x, using Leica Application Suite 3.3.0. CldU and IdU tract lengths were measured using ImageJ. For statistical analyses, Mann-Whitney test was applied to compute if differences in tract lengths were significant using Prism (GraphPad Software).

6.10 Electron microscopic analysis of genomic DNA

In vivo psoralen cross-linking, isolation of total genomic DNA, and enrichment of the replication intermediates and their EM visualization were performed as described in (Neelsen et al., 2014). In brief, cells were harvested, and genomic DNA was cross-linked by two rounds of incubation in 10 μM 4,5′,8-trimethylpsoralen and 3 min of irradiation with 366-nm UV light. Cells were lysed, and genomic DNA was isolated from the nuclei by proteinase K digestion and phenol-chloroform extraction. Purified DNA was digested with PvuII and replication intermediates were enriched on a BND cellulose column. EM samples were prepared by spreading the DNA on carbon-coated grids and visualized by platinum rotary shadowing. Images were acquired on a transmission electron microscope (G2 Spirit; FEI Tecnai) and analyzed with ImageJ (National Institutes of Health).

7. LIST OF ABBREVIATIONS

AP	apurinic/apyrimidic
Ape1	AP endonuclease 1
ASC	adult stem cell
AT	adenine-thymine base pair
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATR	ataxia telangiectasia mutated and Rad3 related
BER	base excision repair
β-ME	beta-mercaptoethanol
BRCA	breast cancer susceptibility protein
BrdU	bromodeoxyuridine
C	cytosine
Cdc	cell division cycle
CDK	cyclin dependant kinase
CFS	common fragile sites
CFSE	carboxyfluorescein succinimidyl ester
CPT	camptothecin
Ctf	chromosome transmission fidelity
DDR	DNA damage response
DDT	DNA damage tolerance
DNA	deoxyribonucleic acid
DNA-PKcs	DNA dependant protein kinase catalytic subunit
Dpb11	DNA polymerase B(II)
DSB	double strand break
EdU	ethynldeoxyuridine
EM	electron microscope
Emi1	early mitotic inhibitor 1
ESC	embryonic stem cell
EU	ethynyluridine
FA	Fanconi anaemia
FACS	fluorescence activated cell sorting

FANCD	Fanconi anaemia group D protein
Gadd	growth arrest and DNA damage inducible
GINS	*go, ichi, ni, san* (Japanese for 5, 1, 2, 3)
GSK3β	glycogen synthase kinase 3 beta
Gy	gray
HR	homologous recombination
HSC	haematopoietic stem cell
HU	hydroxyurea
H1	histone 1
IC	initiation complex
IFN	interferon
IF	immunofluorescence
iPSC	induced pluripotent stem cell
KO	knockout
LIF	leukaemia inhibitory factor
LPS	lipopolysaccharide
Mcm	mini-chromosome maintenance
MEF	mouse embryonic fibroblast
MEK	mitogen activated protein kinase
MSH	MutS protein homologue
NAC	N-acetylcysteine
NAD+	Nicotinamide adenine dinucleotide
NER	nucleotide excision repair
NHEJ	non-homologous end joining
Ola	olaparib
ORC	origin recognition complex
PARP1	poly ADP ribose polymerase 1
PBS	phosphate buffer saline
PFGE	pulse field gel electrophoresis
pI:C	polyinosinic-polycytidylic acid
PI3K	phosphatidylinositol 3-OH-kinase
Pol	polymerase
preRC	pre-replication complex
RB	retinoblastoma

rDNA	ribosomal DNA
RFB	replication fork barrier
RFC	replication fork complex
RI	replication intermediate
RPA	replication protein A
RS	replication stress
Sir2	silent information regulator 2
Sld	synthetically lethal with Dpb11
ssDNA	single stranded DNA
TBM	total bone marrow
TDG	thymine DNA glycosylase
TER	terminator region
Tet	ten eleven translocation factor
Top	topoisomerase
UV	ultraviolet radiation
XPG	*Xeroderma pigmentosum* complementation group G
XRCC1	X-ray repair cross-complementing protein 1
5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine

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9. COLLABORATIVE WORK

During the course of this thesis, I have contributed to various collaborative projects. I have included a submitted manuscript of one such study and the abstract of a nearly finished manuscript for another study. I am co-author on both these studies and have summarized my contribution to the two studies below:

9.1. Poly(ADP-ribosyl) gycohydrolase (PARG) prevents the accumulation of abnormal replication structures during unperturbed S phase

I have substantially contributed to this study, wherein I have performed and analyzed confocal microscopy experiments for localization of DNA damage response (DDR) markers included in figure 4 and S3, DNA double strand break accumulation (DSB) by pulse field gel electrophoresis and DDR signaling by Western blotting included in figure 5. I have also helped in the acquisition and analysis of flow cytometry experiments included in figure 1C, 1D and S1B. In brief, I have shown that PARG deficient cells accumulate increased amounts of the DDR markers γH2AX and Rad51. Further, cells lacking PARG also display numerous 53BP1 foci and active KAP1 signaling, in the absence of any detectable physical DSBs. These experiments complemented other experiments performed in this study and helped in understanding and finalizing the model illustrated in figure 6. The complete manuscript is attached to this thesis.

9.2. PARP-1 inactivation by pyrimidine pool disequilibrium leads to ultrafine anaphase bridge formation

I have performed electron microscopy experiments to assess fork reversal and ssDNA gaps in CDA proficient and deficient cells. These experiments provided mechanistic insights that were crucial in discarding an earlier hypothesis and changing the course of this study. Briefly, I have experimentally shown that CDA deficiency does not lead to extensive uncoupling between the polymerase and the helicase, as was thought earlier. The abstract along with author affiliations is attached to this thesis.

9.1 Poly(ADP-ribosyl) gycohydrolase (PARG) prevents the accumulation of abnormal replication structures during unperturbed S phase

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ABSTRACT

Poly(ADP-ribosyl)ation (PAR) has been implicated in various aspects of the cellular response to DNA damage and genome stability. Although 17 human poly(ADP-ribose) polymerase (PARP) genes have been identified, a single poly(ADP-ribose) glycohydrolase (PARG) mediates PAR degradation. Here we investigated the role of PARG in the replication of human chromosomes. We show that PARG depletion affects cell proliferation and DNA synthesis, leading to replication-coupled H2AX phosphorylation. Furthermore, PARG depletion *per se* slows down individual replication forks similarly to mild chemotherapeutic treatment. Electron microscopic analysis of replication intermediates reveals marked accumulation of reversed forks and ssDNA gaps in unperturbed PARG-depleted cells, which is associated with ATR activation and chromatin recruitment of the ssDNA binding proteins RPA and RAD51. Intriguingly, while we found no physical evidence for chromosomal breakage, PARG depleted cells also displayed enrichment for standard double strand break markers, such as KAP1 phosphorylation and 53BP1 foci. Overall, these data prove PAR degradation essential to promote resumption of replication at endogenous and exogenous lesions, preventing idle recruitment of repair factors to remodeled replication forks. Furthermore, they suggest that fork remodeling and restart are surprisingly frequent in unperturbed cells and provide a molecular rationale to explore PARG inhibition in cancer chemotherapy.

INTRODUCTION

Cellular responses are crucial for the adaptability and survival of a cell to different types of stress-both endogenous and exogenous. The DNA Damage Response (DDR) consists of one such defense mechanism in response to different types of insults to the DNA. Poly(ADP) ribosylation of proteins is one of the first cellular responses to DNA damage which is brought about by proteins of the poly (ADP) ribose polymerase family (PARP), mostly PARP1 (Gibson and Kraus, 2012). Upon being recruited to sites of the DNA damage, NAD^+ is used as a substrate by PARP to synthesize negatively charged poly ADP ribose (PAR) polymers onto itself and also its target proteins (Gibson and Kraus, 2012). Through this post translational modification, PARP targets a variety of nuclear proteins to facilitate the recruitment of DNA repair factors to sites of damage (Bouchard et al., 2003, Woodhouse and Dianov, 2008). PARP-1 or PARP-2 deficient mice and mouse embryonic fibroblast show chromosomal aberrations and various DNA repair defects (de Murcia et al., 1997, Menissier de Murcia et al., 2003, Wang et al., 1997).

Inhibition of PARP is becoming a promising therapeutic approach for the treatment of certain types of cancer (Anders et al., 2010). It was shown that PARP inhibitors could selectively kill homologous recombination (HR) deficient cancer cells (Bryant et al., 2005, Farmer et al., 2005). The reason for this sensitivity of HR deficient cells to PARP inhibition is thought to be the collapse of replication forks running into nicks in the absence of PAR resolving into double stranded breaks (DSB). These DSBs are not repaired due to the absence of HR resulting in the cytotoxicity (Bryant et al., 2005, Petermann et al., 2010). Recently, PARP has been reported to play a role in the control of replication fork reversal upon topoisomerase 1 poisoning (Ray Chaudhuri et al., 2012). The role of PARP in the process has been attributed to its PARylated form interacting and inhibiting the activity of RecQ1 helicase. This would prevent a restart of the reversed forks until repair of the damage has occurred (Berti et al., 2013b).

PAR modification is one of the earliest steps in the DNA damage response and is rapidly degraded by poly(ADP)ribose glycosylase (PARG), an enzyme with both endo and exoglycosidase activities (Gagne et al., 2006, Min and Wang, 2009). PARG has 4 different isoforms in the cells: 99kDa and 102kDa which localize to the cytoplasm, 110kDa which localizes to the nucleus and 60kDa localizing to the mitochondria (Meyer-Ficca et al., 2004). PARG has been shown to involved in controlling a variety of cellular process including apoptosis (Erdelyi et al., 2009, Feng et al., 2012). Depleting all isoforms of PARG in mice results in embryonic lethality (Koh et al., 2004). However, a hypomorphic mutant for the nuclear isoform was viable but highly sensitive to treatments with alkylating agents and ionizing radiation suggesting its role in the maintenance of genome instability (Cortes et al., 2004). PARG depletion has also been shown to result in mitotic catastrophe upon treatments with ionizing irradiation (Ame et al., 2009). Furthermore, PARG is also recruited to sites of DNA damage through PARP and PCNA dependent pathways to facilitate DNA repair (Fisher et al., 2007, Mortusewicz et al., 2011). Recently it was also shown that BRCA2- deficient cells were exclusively sensitive to PARG inhibition and proposing its role in prevention of replication fork collapse (Fathers et al., 2012).

In this work, we show that depletion of PARG results in massive DNA damage accumulation in S-phase. This result in slow fork progression, accumulation of abnormal DNA replication intermediates and recruitment of DNA repair factors in the absence of DSBs. Our results indicate that the DNA damage phenotypes observed upon PARG depletion are a result of abnormal and perturbed replication process in these cells resulting from endogenous DNA damage.

MATERIAL AND METHODS

Materials.

The following antibodies were used: γH2AX (Millipore, # 05-636), 53BP1 (Santa Cruz, sc-22760), CHK1 pS345 (Cell Signaling, #2348), CHK1 (Santa Cruz, sc-8408), KAP1-pS824 (Bethyl, A300-767A), KAP1 (Bethyl, A300-274A), RPA (Calbiochem, NA19L), Rad51 (Santa-Cruz, sc-8349) TFIIH (Santa Cruz, sc-293), β-Tubulin (Santa-Cruz, sc-5274). Camptothecin was purchased from Sigma chemicals.

Cell Lines and culture conditions.

Sh Ctrl and sh PARG SilenciX Hela cells lines were purchased from tebu-bio (http://www.tebu-bio.com/Product/00301-00085). Cell cultures were maintained at 37ºC with 5% CO2 in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and standard antibiotics. (?ug/ml) Hygromycin was added to the medium to provide selection pressure in the SilenciX cell lines.

Proliferation curve.

3 x 10^5 cells were seeded in 10 cm dishes at day 0. After 1,2,3 and 4 days the cells were collected by trypisinization and counted using a Neubauer chamber. The proliferation rate was plotted as a fold change in total cell number with respect to the number of cells seeded at Day 0 using Graphpad Prism software.

Flow cytometry.

For flow cytometric analysis for γH2AX/EdU/DAPI, cells were labeled for 30 min with 10 μM EdU, harvested and fixed for 10 min with 4% formaldehyde/PBS. Cells were washed with 1% BSA/PBS pH 7.4, permeabilized with 0.5% saponin/1% BSA/PBS and stained with anti-γH2AX antibody (Millipore, # 05-636) for 2 hours, followed by incubation with a suitable secondary antibody for 30 minutes. Incorporated EdU was labeled according to the manufacturer's instructions (Invitrogen, # C35002). DNA was stained with 1 μg/ml DAPI, samples were measured on a Cyan ADP flow cytometer (Beckman Coulter) and analyzed with Summit software v4.3.

DNA fiber analysis.

Asynchronous cells were labeled with 30 μM CldU, washed and exposed to 250 μM IdU (±CPT), before collection and resuspension in PBS. Cells were then lysed and DNA fibers stretched onto glass slides, as described (Jackson and Pombo, 1998). The fibers were denatured with 2.5 M HCl for 1 h, washed with PBS and blocked with 2% BSA in phosphate buffered saline Tween 20 for 30 min. The newly replicated CldU and IdU tracks were revealed with anti-BrdU antibodies recognizing CldU and IdU respectively. The secondary antibodies used were anti-mouse Alexa 488 and anti-rat Cy3. Microscopy was done using a Leica DMRB microscope equipped with a Leica DFC360 FX camera. Images were taken at 60× magnification, using Leica Application Suite 3.3.0. Statistical analysis was carried out using GraphPad Prism.

Immunofluorescence staining and Confocal microscopic analysis.

Cells were pre extracted for 10 mins on ice 25mM Hepes pH7.4, 50mM NaCl, 1mM EDTA, 3mM $MgCl_2$, 300mM sucrose and 0.5% TritonX-100 and then fixed using 4% formaldehyde. The cells were then stained with 53BP1, Rad51, RPA and γH2AX antibodies, detected by appropriate secondary antibodies and mounted with Vectashield (Vector Laboratories). Cells were imaged with a Leica TCS Sp5 microscope. Images were taken at 63× magnification, using Leica Application Suite Advanced Fluorescence software. At least 100 cells were analyzed for the statistical analysis.

Western Blot analysis.

Whole cell extracts were prepared in Laemmli buffer(120 mM Tris-Cl pH 6.8, 4% SDS, 20% glycerol); proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblots were carried out using the appropriate antibodies.

DSB detection by PFGE.

DSB detection by PFGE was performed as reported previously (Ray Chaudhuri et al., 2012). Briefly, cells were embedded in a 0.8% agarose plug (2.5×105 cells/plug), digested in lysis buffer (100 mM EDTA, 1% (w/v) sodium lauryl sarcosyne, 0.2% (w/v) sodium deoxycholate, 1 mg/ml proteinase K) at 37 °C for 48 h and washed in 10 mM TrisHCl pH8.0, 100 mM EDTA. Electrophoresis was performed at 14 °C in 0.9% (w/v) Pulse Field Certified Agarose (BioRad) containing Tris-borate/EDTA buffer in a BioRad CHEF DR III apparatus (9 h, 120°, 5.5 V/cm, 30 – 18 s switch time, 6 h, 117°, 4.5 V/cm, 18 – 9 s switch time, 6 h, 112°, 4 V/cm, 9 – 5 s switch time). The gel was stained with ethidium bromide and imaged on an Alpha Innotech Imager.

EM analysis of genomic DNA mammalian cells.

In vivo psoralen cross-linking, isolation of total genomic DNA, enrichment of the replication intermediates and their EM visualization were carried out as described (Neelsen et al., 2014). Briefly, cells were harvested, genomic DNA was crosslinked by two rounds of incubation in 10 μM 4,5',8-Trimethylpsoralen and two minutes of irradiation with 366 nm UV light. Cells were lysed; genomic DNA was isolated from the nuclei by proteinase K digestion and phenol-chloroform extraction. Purified DNA was digested with PvuII and replication intermediates were enriched on a BND cellulose column. EM samples were prepared by spreading the DNA on carbon-coated grids and visualized by platinum rotary-shadowing. Images were acquired on a Philips CM 100 microscope and analyzed with ImageJ.

RESULTS

PARG depletion affects cell proliferation and interferes with unperturbed DNA replication.

To elucidate the functional relevance of PARG activity on chromosome replication, we analyzed a previously established cellular system for stable PARG depletion in Hela cells (Ame et al., 2009 and Supplementary Figure S1A). Our single-cell immunostainings revealed PAR accumulation upon PARG depletion, even in the absence of genotoxic treatments. However, PARG-depleted cells showed small punctate PAR-foci, distinct from the typical pan-nuclear staining observed upon treatment with the genotoxic agent H_2O_2 (Figure 1A). suggesting that PARG depletion leads to PAR accumulation specifically at sites of endogenous DNA damage. Performing cell proliferation assays in shCtrl and shPARG cells, we found that PAR accumulation in shPARG cells is associated with decreased cell proliferation compared to control cells (Figure 1B). We next investigated whether this reduced proliferation reflected problems in chromosomal replication, assessing DNA content (DAPI), DNA synthesis (EdU incorporation) and DNA damage accumulation (H2AX phosphorylation) by flow cytometry (FACS). PARG depletion leads to mild accumulation of S phase cells, accompanied by small but reproducible reduction in EdU incorporation compared to control cells, suggesting that PAR

accumulation interferes with the replication process under unperturbed conditions (Figure 1C). Accordingly, H2AX phosphorylation - reportedly higher upon PARG inactivation (Fathers et al., 2012 and Supplementary Figure S1B) - was specifically increased in cells with intermediate DNA content, thus undergoing chromosomal replication (Figure 1D). Overall, the effects of PARG depletion on EdU incorporation and H2AX phosphorylation were comparable to those induced by mild treatment (25nM) with the prototypical Top1 poison camptothecin (CPT; Figure 1C and D), previously shown to interfere with replication fork progression and to induce fork reversal in the absence of detectable chromosomal breakage (Ray Chaudhuri et al., 2012). Altogether, these data suggest that PARG depletion affects cell proliferation and induces DNA damage by interference with the replication process.

PARG depletion results in slow replication fork progression even in the absence of genotoxic treatments.

These effects on bulk DNA replication in PARG-depleted cells prompted us to test the effect of PARG depletion on the progression of individual replication forks, using a well-estabished "DNA fiber spreading" assay (Jackson and Pombo, 1998). Both shCtrl and shPARG cells were pulse labeled with thymidine analog CldU (detected as red tracks) for 30 min and then for 30 min with a second thymidine analog (IdU, detected as green tracks). Mild CPT treatment was optionally added during the second labeling. In agreement with previous results (Ray Chaudhuri et al., 2012), we confirmed in control Hela cells that mild CPT treatments are sufficient to significantly slow down replication fork progression (Figure 2A and B). Surprisingly, replication forks were slowed down to a similar extent by PARG depletion, even in the absence of exogenous genotoxic treatments. Furthermore, CPT treatment in PARG-depleted cells lead to significant, but quantitatively marginal further effects on the progression of individual forks (Figure 2A and B). Taken together, these data suggest that interfering with PAR catabolism by itself results in marked replication stress and fork slowdown, which is comparable to mild chemotherapeutic treatments.

PARG prevents the accumulation of reversed replication forks and postreplicative ssDNA gaps.

We next investigated whether slow replication fork progression upon PARG depletion was accompanied by altered architecture of replication intermediates, using a combination of in-vivo psoralen crosslinking and transmission electron microscopy (EM; Neelsen et al., 2014). Interestingly, our EM analysis revealed that depletion of PARG in unperturbed cells resulted in a substantial accumulation of reversed replication forks (25% versus 6% in control cells; Figure 3A and B, and Supplementary Figure S2A). The frequency of reversed forks in untreated PARG-depleted cells is close to that observed with mild CPT treatments in control Hela cells (36%, Figure 3B) and that reported upon Top1 poisoning in yeast, Xenopus egg extracts, U2OS and mouse embryonic fibroblasts (15-40%, Ray Chaudhuri et al., 2012). Furthermore, PARG depletion only marginally increased the frequency of reversed forks upon CPT treatments (from 36% to 42%, Figure 3B), mirroring our observations on fork progression by DNA fiber analysis (Figure 2B). In addition to fork reversal, approximately 50% of the replication forks in PARG-depleted cells exhibited ssDNA gaps on

replicated duplexes, compared to about 20% of the forks in control cells (Figure 3B and D, and Supplementary Figure S2B). A significant fraction of replication intermediates from PARG-depleted cells displayed 2 or more ssDNA gaps, detectable on both replicated duplexes (Figure 3B and Supplementary Figure S2B). As shown for fork slowing and fork reversal, CPT treatment did not markedly increase ssDNA gap accumulation in PARG depleted cells (Figure 3B). Overall, these EM data show that PARG depletion in unperturbed cells results in alterations of replication fork structure that resemble mild chemotherapeutic treatments.

PARG prevents recruitment of DSB repair factors to replicating chromatin.

Since PARG depletion results in profound structural alterations of replication intermediates and H2AX phosphorylation, we next tested whether this was accompanied by detectable chromatin recruitment of other DDR factors, previously involved in the response to DSB. Our IF-based confocal experiments confirmed our FACS results (Figure 1D) and the previously reported accumulation of γH2AX foci in unperturbed PARG depleted cells (Fathers et al., 2012; Figure 4A). Surprisingly, we noticed that a large fraction of γH2AX-positive cells upon PARG depletion was accompanied by co-localization of the DSB repair factor 53BP1, both in absence and presence of exogenous genotoxic stress (CPT; Figure 4A-C). Besides the fraction of 53BP1-positive cells (cells with more than five foci), PARG depletion clearly increased the number of γH2AX/53BP1 foci detected in these positive cells, leading to a dense, punctuated immunostaining pattern that resembles control cells treated with mild CPT doses (Figure 4A and B). Importantly, these small 53BP1 foci detected by confocal microscopy are clearly distinguishable from the intense larger 53BP1 foci observed in IR-treated cells (Supplementary Figure S3A and B) and may escape detection by epifluorescence microscopy (Ray Chaudhuri et al., 2012). One possible interpretation of these data is that PARG depletion, similarly to low CPT doses, leads to mild and transient chromosomal breakage, marked by local 53BP1 recruitment. In line with this hypothesis, one additional marker typically recruited to DSB upon end resection - i.e. RAD51 - displayed similar trends of accumulation upon PARG depletion, particularly evident in terms of number of detected foci (Figure 4B and E and Supplementary Figure S3C). As for 53BP1 recruitment, PARG depletion *per se* was sufficient to induce RAD51 recruitment at similar levels as mild CPT treatments (Supplementary Figure S3C). Taken together, these data indicate that DNA replication interference by PARG depletion is associated with recruitment of DDR and DNA repair factors to DSB or other replication-associated DNA structures.

Depletion of PARG results in checkpoint activation uncoupled from detectable DSB formation.

In line with the hypothesis that unusual DNA structures resembling DSB may accumulate upon PARG depletion, promoting the recruitment of repair factors, we observed that PARG depleted cells display activated ATR/Chk1 and ATM/Kap1 pathways in the absence of exogenous damage, although mild CPT treatment was required to have these molecular events at comparable levels with IR-treated cells (Figure 5A). Using an optimized pulsed field gel-electrophoresis (PFGE) protocol, which can detect < 100 DSB per cell (Berti et al., 2013b, Ray Chaudhuri et al., 2012), we verified that ATM/ATR activation upon IR-treatment is associated with marked accumulation of DSB. However, PARG depletion, even

in combination with mild CPT treatments, was not associated with detectable chromosomal breakage over background levels (Figure 5B). These surprising results uncouple DSB formation from ATM/ATR signaling and 53BP1/RAD51 recruitment, and strongly suggest that checkpoint activation and recruitment of repair factors under these experimental conditions reflect accumulation of unusual DNA structures different from DSB.

DISCUSSION

In this study, we show that depletion of PARG in Hela cells results in reduced cell proliferation associated with impairment of the DNA replication process. This is accompanied by several recognized markers of replication stress, spanning from H2AX hyper-phosphorylation in S phase to impaired progression of individual replication forks and ATR/ATM activation. Notably, impaired replication fork progression is accompanied by a widespread alteration of fork structure, most notably accumulation of post-replicative ssDNA gaps and reversed replication forks. Accumulation of these unusual structures during replication is accompanied by chromatin recruitment of DDR and DSB repair factors, in the absence of detectable chromosomal breakage. Importantly, albeit exacerbated by genotoxic treatments, all these phenotypes are already clearly detectable in unperturbed PARG depleted cells, clearly showing that PAR catabolism is of crucial importance to assist complete and effective replication during unperturbed S phase.

PARP activity was recently reported to mediate accumulation of reversed replication forks upon Top1 inhibition (Ray Chaudhuri et al., 2012), by transiently inhibiting the fork restart activity of the RecQ1 helicase (Berti et al., 2013b), in order to coordinate fork restart with repair of the damaged template. While PARG depletion was therefore expected to induce reversed fork accumulation upon CPT treatment, the drastic accumulation of reversed forks and the global reduction of fork speed in untreated PARG-depleted cells are important unexpected observations. These data strongly suggest that endogenous lesions and/or alternative DNA structures are inducing transient reversal of replication forks at a high number of locations in unperturbed S phase and require active PAR degradation to ensure continued replication fork progression once the obstacles have been overcome (Figure 6). This is in agreement with the recent finding that repetitive DNA sequences with a propensity to form non-B DNA structures induce reversal of traversing replication forks at remarkably high frequencies (Follonier et al., 2013). Considering the high number of endogenous DNA lesions (refs) and the abundance of non-B forming structures in the human genome (refs), it is conceivable that fine-tuning of PAR synthesis and degradation at a number of chromosomal locations plays a pivotal role in assisting complete and faithful replication of the human genome. These observations may contribute to explain the mitotic defects previously associated with the essential role of PARG in development, as deletion of all isoforms of PARG leads to embryonic lethality in mice (Follonier et al., 2013).

Recently PARG inhibition was shown to kill homologous recombination (HR)-defective cells, via a replication-dependent mechanism (Fathers et al., 2012). Although the effect of PARG inhibition on the replication process was not directly investigated, the authors postulated that replication fork collapse by PARG inhibition may lead to DSB formation and thus HR requirement to restart collapsed forks.

We now show that PARG depletion affects the progression of all replication forks and alters the molecular architecture of a significant fraction of replication intermediates, in the absence of detectable chromosomal breakage. We also show that accumulation of these unusual replication intermediates is accompanied by DDR activation and chromatin recruitment of DSB repair factors. Not all the experimental conditions recently reported to induce replication fork reversal have been associated with DDR activation (Ray Chaudhuri et al., 2012), excluding that the physiological reversal process, with the formation of a DNA end (regressed arm) at replication intermediates, is *per se* sufficient to induce checkpoint activation and chromatin recruitment of DSB repair factors. We propose that the persistence of these DNA structures and/or their associated processing upon impairment of PAR degradation may lead to recruitment of cellular factors usually recruited at DSB, either before or after their nucleolytic processing (53BP1 and RAD51, respectively; Figure 6). It is possible that recruitment of these factors and ATR/ATM checkpoint activation are linked to minor changes in the molecular architecture of reversed forks, associated with their persistence, which may escape detailed EM visualization. In this scenario, upon PARG depletion and reversed fork accumulation, HR and other DSB repair factors may become essential to drive alternative, RecQ1-independent pathways for the restart of reversed replication forks, which may provide an alternative explanation for the reported requirement of HR for cell survival upon PARG inhibition (Fathers et al., 2012). Detection of ssDNA gaps have been linked in model systems to repriming events across DNA lesions (Hashimoto et al., 2010a, Lopes et al., 2006). Although we cannot directly link the observed accumulation of post-replicative ssDNA gaps to the persistence of reversed forks, a tantalizing alternative hypothesis is that ssDNA gaps may accumulate on replicated duplexes as a consequence of these RecQ1-independent fork restart event (Figure 6), which may entail nucleolytic degradation rather than branch migration of the reversed forks (Berti et al., 2013b). In agreement with this model, nucleolytic processing of reversed forks has been previously reported in yeast (Cotta-Ramusino et al., 2005, Hu et al., 2012). Regardless of their source, postreplicative ssDNA gaps can certainly contribute to explain the observed accumulation of RAD51 in PARG-depleted cells. Intriguingly, RAD51 was previously reported to limit ssDNA accumulation at yeast and *Xenopus* replication forks, especially in response to genotoxic stress (Hashimoto et al., 2010a). Furthermore, RAD51 itself and several HR and Fanconi Anemia factors, were shown to prevent excessive degradation of newly synthesized DNA in response to replication stress (Schlacher et al., 2011a, Schlacher et al., 2012a). Whether the role of HR factors in face of endogenous or exogenous replication stress is related to replication fork remodelling will be subject of intense studies in the near future.

Altogether, showing that PAR degradation is required for remodelling of replication forks in unperturbed S phase, our data provide mechanistic insight on the essential role of PARG in cell growth and development. At the same time, by showing that the molecular defects associated with PARG inactivation are exacerbated by mild chemotherapeutic treatments, we provide a molecular basis for the anticipated use of PARG inhibitors to potentiate cancer chemotherapy (Tentori et al., 2005). This attractive therapeutic perspective has been so far hampered by the limited specificity of the currently available PARG inhibitors (Min and Wang, 2009), which will be likely improved based on the recent resolution of PARG crystal structure (Slade et al., 2011). As at least one additional protein -

i.e. the ADP-ribose protein glycohydrolase TARG1 - has been recently shown to assist PARG in full removal of PAR chains from target proteins and has been implicated in human disease (Sharifi et al., 2013), it will be important to test the possible involvement of this and possibly other PAR degrading enzymes in the maintenance of genome stability during replication.

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The quick brown fox jumps over the lazy dog. The quick brown fox jumps over the lazy dog. The quick brown fox jumps over the lazy dog. The quick brown fox jumps over the lazy dog. The quick brown fox jumps over the lazy dog.

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FIGURE LEGENDS

Figure 1. PARG depletion results in reduced proliferation and accumulation of DNA damage in replicating human cells. (A) Immunofluorescence analysis of shCtrl and shPARG Hela cells for formation of Poly(ADP) ribosylation (PAR). 1mM H_2O_2 (10min) treatment was used as a positive control. (B) Proliferation curve of shCtrl and shPARG Hela cells. The proliferation rate has been plotted as fold change in cell number with respect to number of cells seeded at Day 0. (C) Flow cytometric analysis of DNA synthesis (EdU), DNA content (DAPI) in shCtrl and shPARG Hela cells with mock (NT) or camptothecin (CPT) 25nM treatment. The percentage of cells in S-phase is indicated in brackets. The green dashed line indicates maximal EdU incorporation in control cells, while the red dashed line indicates reduced maximal incorporation upon PARG depletion and/or CPT treatment. Blue dots indicate cells scored positive for γH2AX, as measured in D. (D) Flow cytometric analysis of DNA content (DAPI) and H2AX phosphorylation in shCtrl and shPARG cells with mock (NT) or camptothecin (CPT) 25nM treatment. The green dashed line indicates the threshold set for γH2AX positivity, while the red dashed line indicates maximal γH2AX levels detected upon PARG depletion and CPT treatment. A mild increase in H2AX phosphorylation is detected during replication also in control Hela cells, but γH2AX levels are clearly increased upon PARG depletion.

Figure 2. PARG depletion slows down replication fork progression. (A) Schematic experimental conditions for DNA replication track analysis. shCtrl and shPARG cells were labelled with CldU and IdU as indicated. Red and green identify CldU- and IdU-containing tracks, respectively. 25 nM CPT was optionally added concomitantly with the second label. Representative DNA fiber tracks from shCtrl and shPARG cells with or without CPT treatment are shown below. White scale bar: 5 μm. (B) Statistical analysis of IdU tract length measurements from shCtrl or shPARG cells. Relative length of IdU tracts (green) synthesized after mock (NT) or CPT treatment (50nM). At least 125 tracks were scored for each dataset. Whiskers indicate the 10 - 90 percentiles. Statistical test according to Mann-Whitney, results are *p=0.0206, *** p<0.0001.

Figure 3. PARG depleted cells accumulate reversed forks and ssDNA gaps on replicated duplexes (A) Representative electron micrograph of a reversed fork observed on genomic DNA from shPARG untreated cells. The black arrow points to the four-way junction at the replication fork, indicative of fork reversal. (B) Frequency of fork reversal in shCtrl and shPARG Hela cells treated with or without CPT (25 nM). In brackets, the number of analyzed molecules. (C) Representative electron micrograph of a replication fork observed on genomic DNA from shPARG untreated cells. The white arrows point to ssDNA gaps along the replicated duplexes, detectable by locally reduced thickness of the DNA filament (Neelsen et al., 2014). (D) Statistical distribution of the number of ssDNA gaps observed in the same populations of molecules analyzed in (B). Very similar values to B and D have been obtained in another independent EM experiment.

Figure 4. PARG depletion leads to chromatin accumulation of typical DSB markers. (A) and (B) Representative images from confocal immunofluorescence analysis of shCtrl and shPARG cells treated with or without 25 nM CPT and stained for γH2AX and 53BP1(A) and Rad51(B).

Representative images are shown for each condition. (C) and (D) Quantification plots show the percentage of gH2AX positive cells also positive for 53BP1 or Rad51 respectively.

Figure 5. Checkpoint activation upon PARG depletion can be uncoupled from DSB formation. (A) PFGE analysis of DSB formation in shCtrl and shPARG Hela cells optionally treated with CPT 25nM. 20Gy IR treatment was used as a positive control for DSB. (B) Western blot analysis of ATR- and ATM-checkpoint activation (CHK1- and KAP1-phosphorylation respectively) in shCtrl and shPARG Hela cells optionally treated with CPT 25nM. 20Gy IR treatment was used as a positive control for pKAP1 and pChk1 induction. GAPDH was used a loading control.

Figure 6. Model suggesting the role of PARG in the maintenance of unperturbed DNA replication. In normal cells, replication forks encountering endogenous lesions undergo dynamic fork reversal and fork restart mediated PARG thus maintaining genome integrity. However in the absence of PARG, forks reversed at endogenous lesions fail to restart due to lack of PAR degradation. Failure to restart reversed forks leads to unscheduled recruitment of DNA repair factors resulting in pathological DNA structures, impaired replication and genome instability.

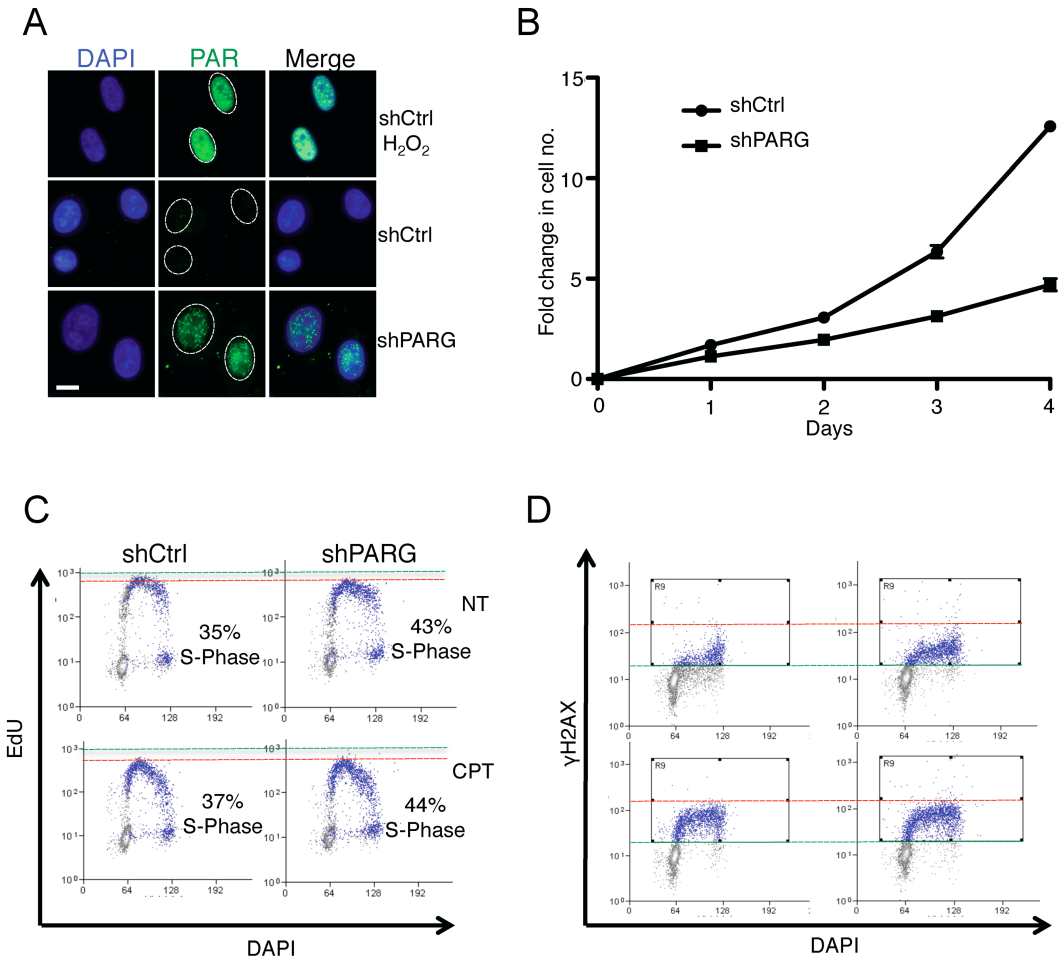
SUPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1. (A) Western blot analysis of PARG protein in shCtrl and shPARG Hela cells. Tubulin has been used as loading control. (B) Quantification of γH2AX positive cells in shCtrl and shPARG cells with mock or CPT treatment, based on the flow cytometry data shown in Figure 1C and D.

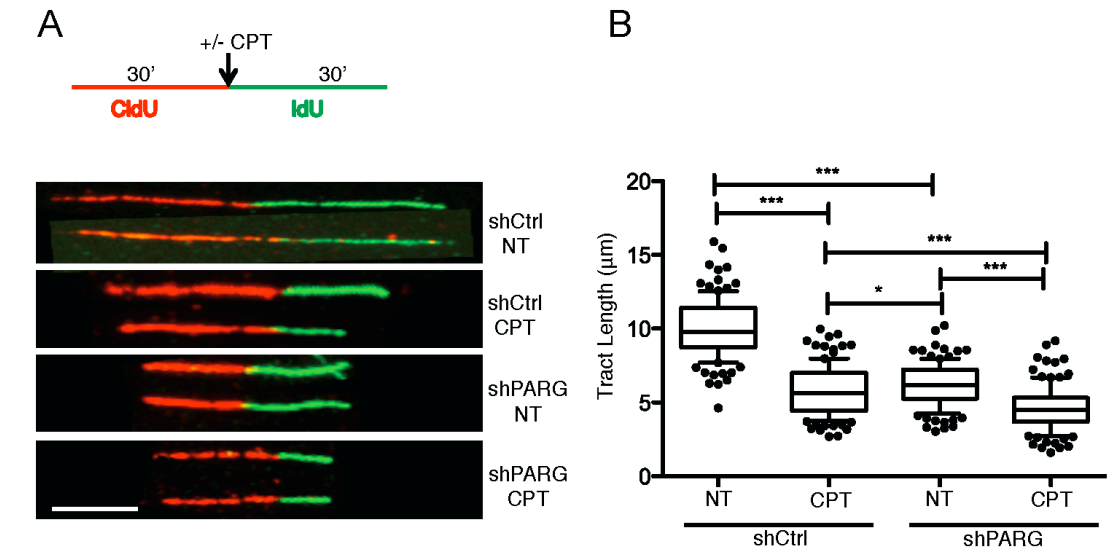
Supplementary Figure S2. (A) Additional representative electron micrograph of a reversed fork observed on genomic DNA from shPARG untreated cells. The black arrow points to the four-way junction at the replication fork, indicative of fork reversal. (B) Additional representative electron micrograph of replication fork observed on genomic DNA from shPARG untreated cells. The white arrow points to ssDNA gaps along the replicated duplexes.

Supplementary Figure S3. (A) Representative images for immunofluorescence analysis of shCtrl and shPARG treated with 10Gy IR and stained for γH2AX and 53BP1. (B) Quantification plots showing the percentage of γH2AX positive cells also positive for 53BP1 in (A). (C) Representative images for immunofluorescence analysis of shCtrl and shPARG treated with or without 25 nM CPT and stained for γH2AX and Rad51

Ray Chaudhuri et al., Figure 1



Ray Chaudhuri et al., Figure 2



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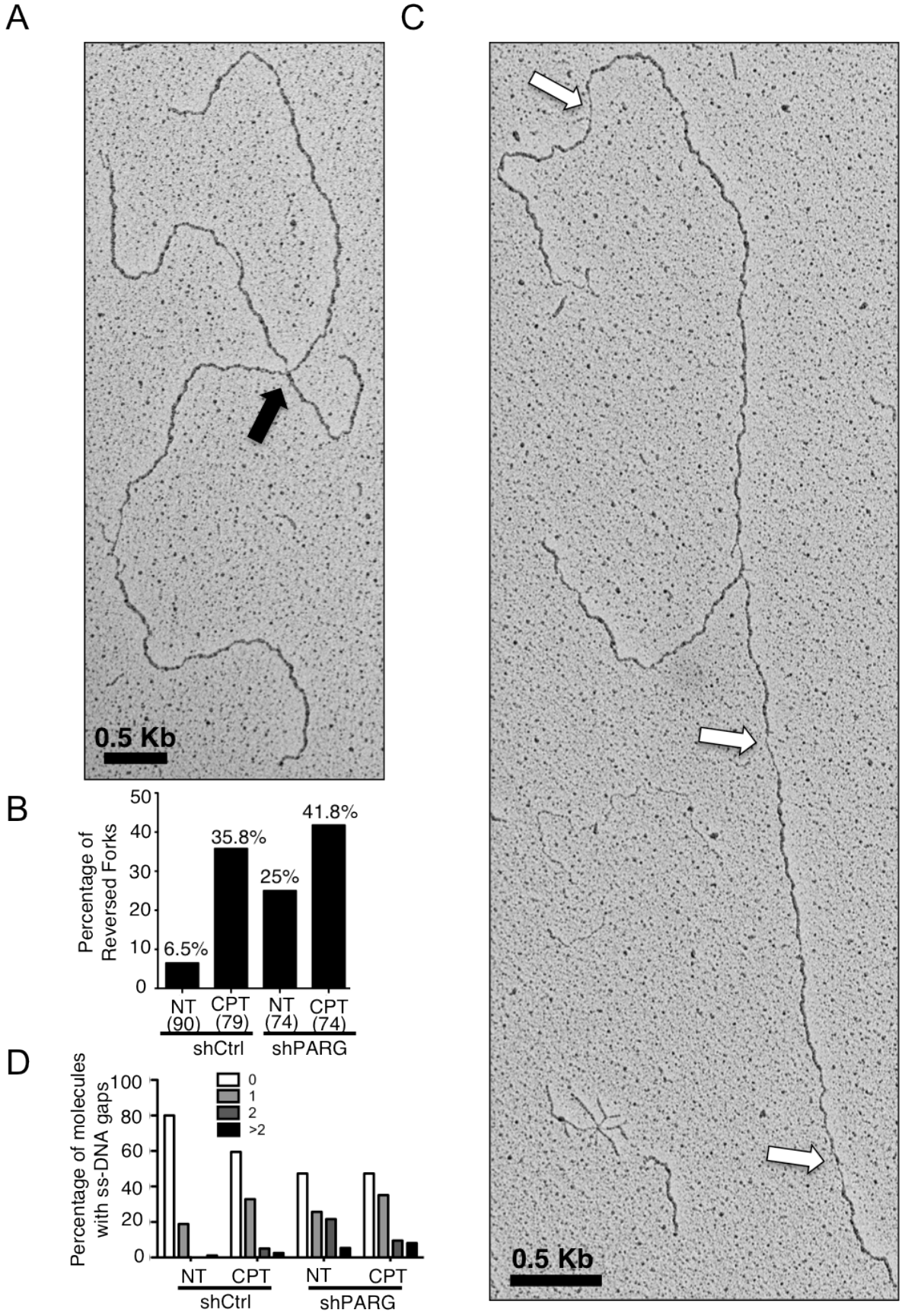

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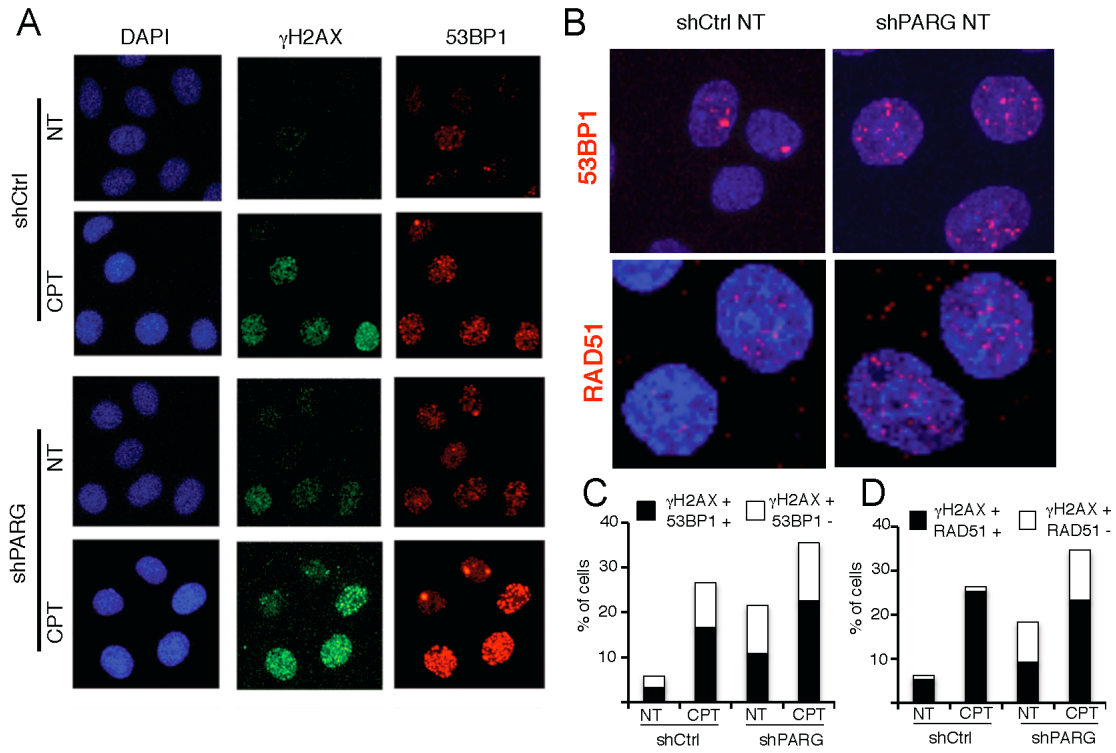

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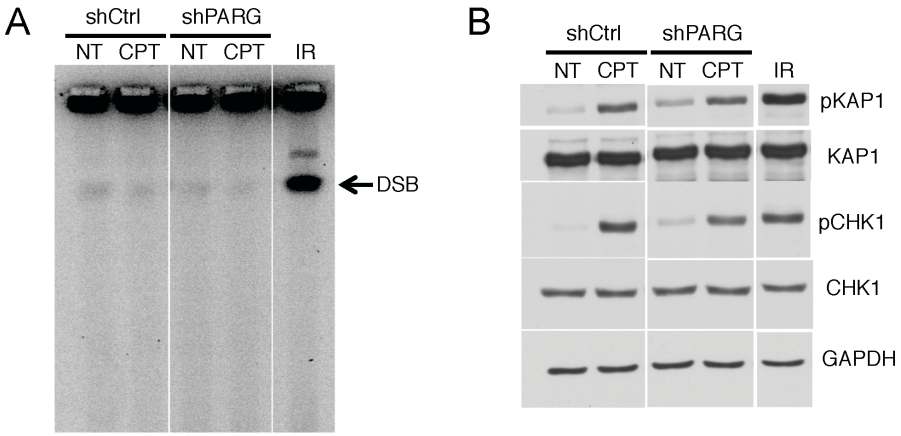

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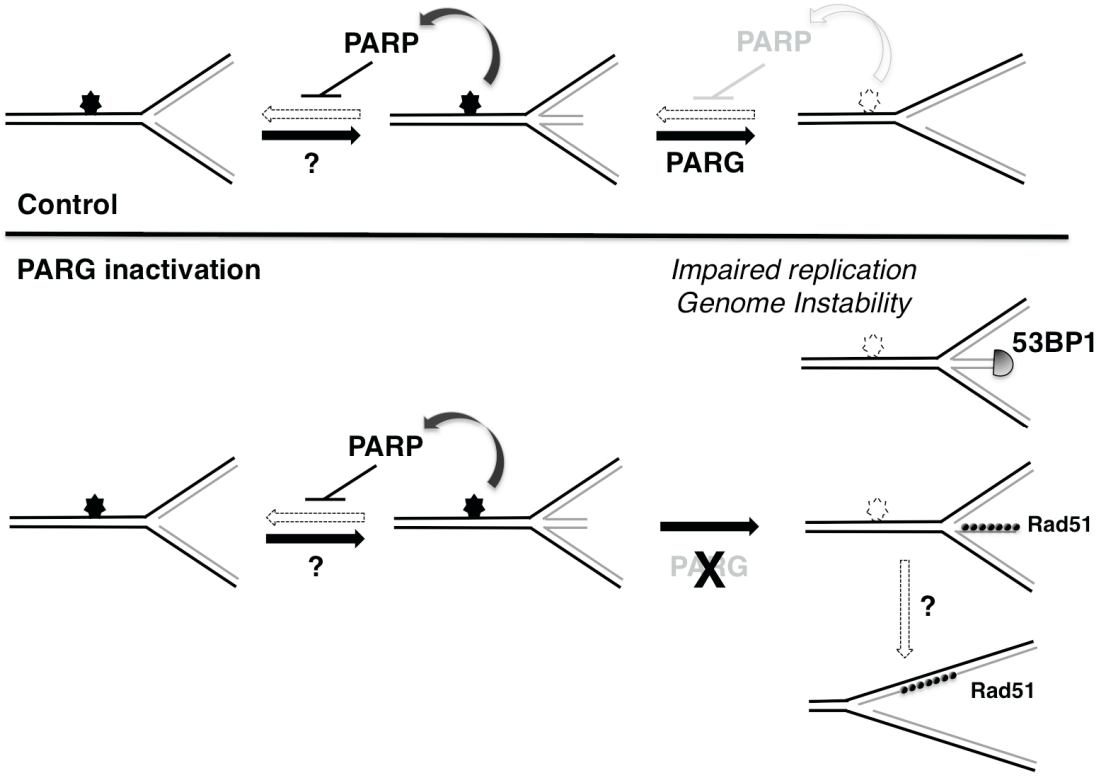
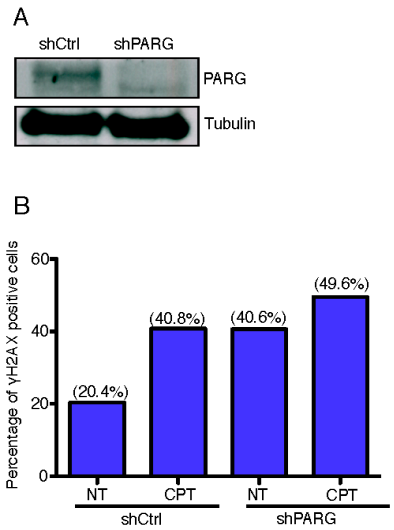
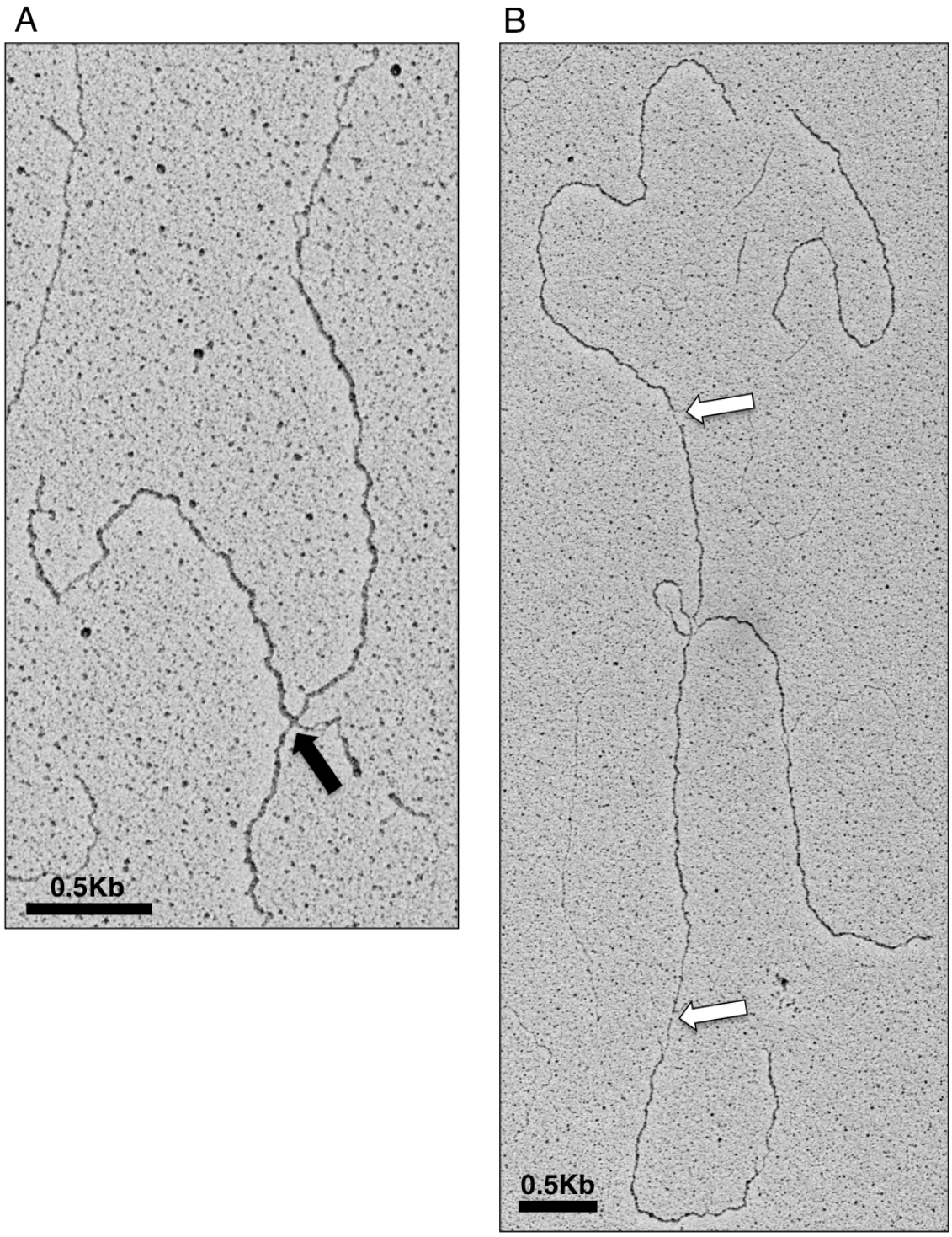


Ray Chaudhuri et al., Supplementary Figure S1



Ray Chaudhuri et al., Supplementary Figure S2



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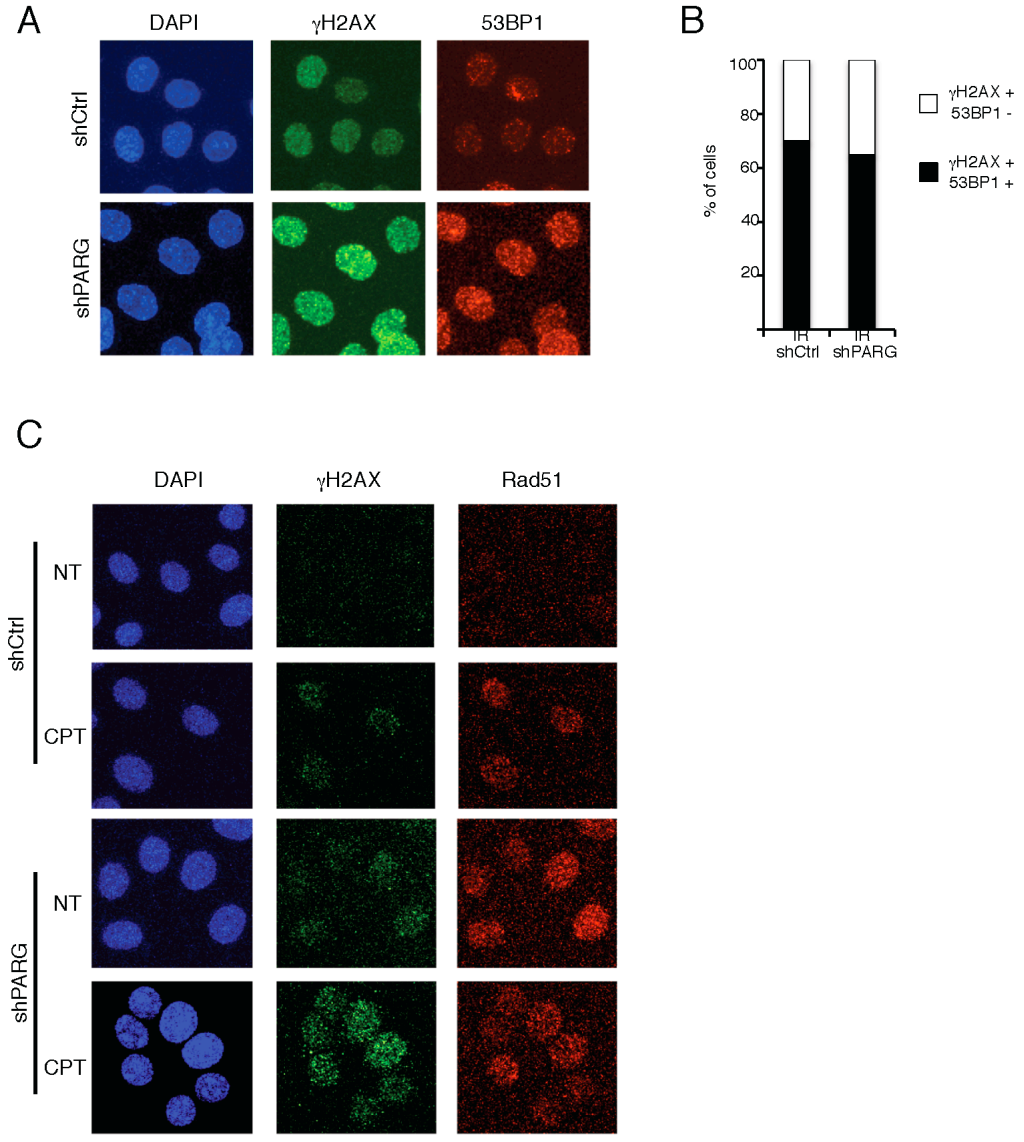

9.2 PARP-1 inactivation by pyrimidine pool disequilibrium leads to ultrafine anaphase bridge formation

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Ultrafine anaphase bridges (UFBs) are DNA structures coated by two helicases, PICH and BLM. Supernumerary UFBs are induced under stress conditions, such as BLM deficiency that leads to Bloom syndrome (BS), a cancer-prone disease with genetic instability. Recent work has shown that BLM deficiency is associated with a cytidine deaminase (CDA) defect, leading to a pyrimidine pool disequilibrium that is responsible for the slowing down of replication fork velocity in BS cells. The relationship between BLM deficiency and supernumerary UFBs is unknown. Here we report that the nucleotide pool disequilibrium due to CDA deficiency is responsible for the increase in UFB frequency in BLM-deficient or -proficient cells. CDA depletion leads to an increase in mitotic DNA synthesis and UFB-containing unreplicated DNA that does not result from replication uncoupling. Mitotic defects are due to partial inactivation of PARP-1 resulting from the excess of dCTP due to the unbalanced nucleotide pool. PARP-1 activation fully rescues both mitotic DNA synthesis and UFB frequency. These data identify dCTP as an inhibitor of basal PARP-1 activity and reveal an unexpected link between PARP-1 and UFBs.

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On a personal note, I extend my deepest regards and love to my parents for shaping me into the individual that I have become. Heartfelt thanks to my siblings for cheering me up, to my partner for bearing with me through this demanding period and to all my friends who have helped me keep my spirits high.

I would like to end with a picture (taken in front of my house in Zurich) that has reminded me to pause, think, remove obstacles from the path up ahead, and continue to move forward. This, I think, is reminiscent of a particular structure that frequently arises during DNA replication in mouse embryonic stem cells.